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ERRATA

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Page 6, line 36: Omit "(basic)."
Page 7, line 1: For "in that the mercuric ion is the cation," read *in that the mercury is present in the cation.*
Page 23, line 20: Omit "and John L. White."
Page 24, line 3: For "90.6" read *83.6.*
Page 24, line 9: For "33.8, 30.4, 14.5" read *72.9, 75.4, 81.0.*
Page 24, line 10: After "90.1," insert *70.4, 42.4.*
Page 24, line 11: After "82.3," insert *96.4.*
Page 24, line 17: For "5400" read *6300.*
Page 33, line 19: For "Among 60 controls 5 positive" read *Among 54 controls 6 positive.*
Page 40, line 9: Omit "(now in press)."
Page 40, line 25: For "Five out of 22 strains" read *Six out of 28 strains.*
Page 40, line 27: For this paragraph read *In a collection of over 300 strains of hemolytic streptococci from a great variety of human diseases a few agreed with certain of the streptococci from strangles.*
Page 48, line 16: For "669" read *615.*
Page 50, lines 11 and 12: For these lines read *some union takes place locally between the tissue and the antigen. This union is probably of the same colloid chemical nature as the union between immune serum and antigen. The capacity.*
Page 50, line 38: For "the" read *an.*
Page 51, line 16: Omit "of a tissue of an immunized animal."
Page 51, line 17: For "A method which showed" read *One established.*
Page 51, line 19: Omit "above."
Page 55, line 6: For "+0.19, pH 7.4" read *+0.18, pH 7.6.*
Page 55, line 8: For "−0.13" read *+0.18.*

SCIENTIFIC PROCEEDINGS

THIRTY-SIXTH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CHICAGO, ILLINOIS, DECEMBER 27, 28 AND 29, 1934

Headquarters: Drake Hotel

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ABSTRACTS*

Report of the Committee on Bacteriological Technic: Progress During 1934.

H. J. Conn, Chairman; Victor Burke, Ivan C. Hall, M. W. Jennison, J. A. Kennedy, Barnett Cohen and Elizabeth F. Genung, *Members.*

During 1934 the Committee has revised three leaflets of the Manual of Methods for Pure Culture Study of Bacteria and has issued a new index to the Manual. The leaflets revised have been Nos. IV, V and VI, dealing, respectively, with Staining Procedures, Routine Methods for the Descriptive Chart, and Biochemical Procedures. All of these revisions have been published in the journal *Pure Culture Study of Bacteria*, which is issued quarterly as a continuation service for the Manual.

Early this year a new member of the Committee, M. W. Jennison, was appointed and has been made primarily responsible for the Society's Descriptive Chart. Under his supervision, work is now under way looking toward a very considerable change in this chart. A preliminary draft of the new revision is now ready for the Society's consideration; how soon to put the new edition on sale will depend upon what further suggestions for revision are received.

In considering the business side of the Committee's work, one may well conclude that the depression is over. During the year 1933, 22,947 Descriptive Charts were sold and 294 copies of the Manual. During the 10 months of 1934 ending November 1, the number of Charts sold has been 25,308 and the number of Manuals 284,—in other words, more Charts and nearly as many Manuals as during the entire year of 1933. The royalty turned in to the Society during 1933 was $333.70; while in 1934 the royalty, without including the last two months, has been $345.00. One reason for the larger royalty is because in 1933 only 10 per cent was turned in on the journal, *Pure Culture Study of Bacteria;* but as this is now on a much better established basis, 25 per cent of the subscription receipts have been turned in, as well as 25 per cent of the sales of Charts and Manuals. The money thus turned into the Society's treasury amounts to about 50 cents for each member of the Society, and

* This number of the Journal has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, due to restrictions of time imposed by the printing schedule.

it is hoped that the continued prosperity of this business may help make it possible for the Council of the Society to postpone still further the increase in dues which has been expected for the last few years.

GENERAL BACTERIOLOGY

G1. Bacteria in Travertine from the Yellowstone. CHAS. B. LIPMAN, University of California, Berkeley.

The work described in this paper concerns an extension of former studies of bacteria in rocks and in other old materials, such as adobe brick, old soils, etc., in which a search was made for microorganisms which have survived for long periods of time in a living condition. The method used for making the study on typical samples was to place a block of travertine from Terrace Mountain, which represents a long series of extinct calcareous hot springs, into molten paraffine at a temperature of about 200°C. for about 3½ minutes. The block was then broken open with sterile tools and pieces from the heart of the block were placed in a sterile moist chamber. The moist chamber was then transferred to a sterile inoculation chamber and pieces taken therefrom were flamed for 15 to 20 seconds in a flame with a temperature varying from 1300° to 1600°C. It was then instantly dropped into a sterile mortar and crushed. The crushed powder was then distributed into a variety of media and the bacteria were studied. Results of these studies show that there are filamentous bacteria of a remarkable and unique type in the travertine, which grow in travertine extract containing 1 per cent peptone and in travertine extract plus 1 per cent peptone plus soil extract. A variety of other media inoculated with the crushed travertine have not thus far yielded any growth. These bacteria are described, and photomicrographs accompany the paper.

G2. Stalked Bacteria, a New Order of Schizomycetes. ARTHUR T. HENRICI AND DELIA JOHNSON, University of Minnesota, Minneapolis.

A study of periphytic bacteria upon glass slides immersed in freshwater habitats shows the general occurrence of a group of bacteria, hitherto almost unknown, which secrete stalks by which they are attached to a firm substrate. *Gallionella ferruginea* Ehrenberg and *Nevskia ramosa* Famintzin are stalked bacteria previously described which are quite distinct from the forms we have observed. *Pasteuria ramosa* Metchnikoff, or a very similar form, has been rediscovered. We can add to these a variety of new forms, and propose the creation of a

new order, the **Caulobacteriales,** to include all stalked bacteria. We suggest the division of this order into 4 families and 5 genera according to the following key:

A. Long axis of cell transverse to long axis of stalk; stalks dichotomously branched.
  I. Stalks lobose, composed of gum, forming zoögloea-like colonies. Family **Nevskiaceae** Genus *Nevskia*
  II. Stalks are twisted bands of ferric hydroxide. Family **Gallionellaceae** Genus *Gallionella*
B. Long axis of cell coincides with axis of stalk; stalks slender, filamentous.
  I. Reproducing by transverse fission, stalks unbranched. Family **Caulobacteriaceae** Genus *Caulobacter*
  II. Reproducing by longitudinal fission and by budding; stalks often branched in whorls. Family **Pasteuriaceae**
    a. Stalks very short or lacking, cells sessile. Genus *Pasteuria*
    b. Stalks long and slender. Genus *Blastocaulos*

Several species have been grown in crude cultures in the dark. Their distribution indicates that they are heterotrophic aerobes. Their minute size and structureless cells indicate that they are bacteria. While resembling the **Chlamydobacteriales,** they are neither filamentous nor ensheathed.

G3. The Relation of von Hibler's "Bacillus VI" to Bacillus carnis (Klein) and Its Differentiation from Other Microaerophilic Bacilli. NICHOLAS D. DUFFETT AND IVAN C. HALL, School of Medicine and Hospitals, Univ. of Colorado, Denver.

Four strains of von Hibler's *"Bacillus VI"* (1908), originally described as *"Bacillus V"* in 1899, were studied with reference to morphologic, cultural, pathogenic, and serologic properties. Three of these strains were received as pure cultures; one was contaminated by *Corynebacterium lymphophilum* (Torrey, emend, Bergey, *et al.*). All of the pure strains of von Hibler's *"Bacillus VI"* were shown to be identical and to constitute a distinct species of microaerophilic bacilli.

Von Hibler's *"Bacillus VI"* was identified with *Bacillus carnis* (Klein). The latter name should be used in preference to *Hiblerillus sextus* (Heller) and *Bacillus lactiparcus* (Lehmann and Süssmann) on the ground of priority.

The following observations of Zeissler relating to von Hibler's *"Bacil-*

lus VI" are hereby confirmed and restated in terms of *Bacillus carnis*: (a) *Bacillus carnis* was a pathogenic, non-proteolytic, component of a culture of "*Bacillus enteritidis sporogenes*" sent by Klein to von Hibler and described by the latter as "*Bacillus IV*"; (b) *Bacillus carnis* grows well under anaerobic and delicately under aerobic conditions with production of gas in the ordinary liquid and semisolid mediums. Spores are subterminal. Deep iron brain medium is not blackened. Gelatin and Löffler's coagulated serum are not liquefied. Gas is formed in milk, but the medium is not coagulated. Glucose, galactose, levulose, sucrose, lactose, maltose and salicin are fermented. Glycerol, mannitol, dulcitol, rhamnose, and inulin are not fermented; (c) *Bacillus carnis* produces a weak filterable exotoxin; (d) Highly specific agglutinins are produced in rabbits as a result of subcutaneous injections of *Bacillus carnis* cultures.

Bacillus carnis is hemolytic when grown aerobically or anaerobically on plain or on glucose blood agar.

Agglutinins were produced with difficulty using young toxic cultures, but easily using old non-toxic cultures.

Specific antiserums were produced which neutralized all of the cultures of *B. carnis*, but not those of other microaerophilic and pathogenic anaerobic bacilli.

Bacillus carnis differs from *Bacillus histolyticus* in its failure to blacken iron brain, to produce tyrosine, and to liquefy gelatin, and in its ability to ferment glucose and lactose; it differs from *Bacillus tertius* in having subterminal spores, and being non-hemolytic and pathogenic, and in its failure to ferment mannitol.

G4. A Study of Gram Negative Bacilli of the Genus Bacteroides. LUTHER THOMPSON AND J. C. HENTHORNE, Mayo Clinic, Rochester, Minn.

Gram negative, anaerobic, non spore-forming bacilli have been isolated from various human lesions. Four strains resemble the description of *Bacillus fragilis* (Veillon and Zuber). Three of these strains are similar, but the fourth strain differs from the others slightly in sugar fermentation and agglutination reaction. The strains designated as *Bacteroides fragilis* had been isolated from a mixed flora in a pelvic abscess, a sacral sinus, and two suppurative appendices.

Another species of *Bacteroides* has readily been identified with the description of *Bacillus funduliformis*. *Bacteroides funduliformis* had been isolated in pure culture from four liver abscesses, a colostomy, and from a lung abscess.

Bacteroides funduliformis and *Bacteroides fragilis* are easily differentiated by morphology, fermentation reactions, hemolysis of red cells and agglutination.

Another strictly anaerobic organism systematically studied in this group is a Gram negative bacillus which characteristically produces large amounts of gas on carbohydrate media.

G5. The Bactericidal and Antiseptic Action of Preservatives Frequently Used in Biological Products, and the Effect of These Preservatives on the Potencies of the Products. CAROLYN ROSENSTEIN AND IDA LEVIN, assisted by HATTIE LEVIN, Bureau of Laboratories of the Department of Health, City of New York.

A study of the bactericidal and antiseptic action of preservatives frequently used in biological products and the effect of these preservatives on the potencies of the products showed that no one of these preservatives alone completely controls all the types of contamination found in biological products. This is particularly true in the case of serums and antitoxic-globulins. It was further found that half quantities of phenol and merthiolate in combination (0.25 per cent phenol and 1–20,000 merthiolate) successively added to serums, antitoxic-globulins, and toxins destroyed diphtheroids, *B. pyocyaneus*, and staphylococci, whereas 0.5 per cent phenol did not destroy diphtheroids and 1–10,000 merthiolate did not destroy *B. pyocyaneus*, when these preservatives were used singly.

The bactericidal action of phenol and tricresol has a tendency to decrease after prolonged storage (ten months) while that of merthiolate and the combination of phenol and merthiolate was apparently unaffected. The potency of the products was not affected by 10 months of storage at 5°C. with phenol, tricresol, merthiolate, or the combination of phenol and merthiolate in the dilutions used in these tests.

It was found also that it is necessary to use both solid and liquid media in order to detect all the types of contamination which may be present in biological products.

G6. A Study of the Bacteriostatic and Germicidal Powers of Merphenyl Nitrate. ORTON K. STARK AND MARJORIE MONTGOMERY, Miami University, Oxford, Ohio.

Merphenyl nitrate (basic) is the double salt of phenylmercuric hydroxide and the normal phenylmercuric nitrate. It is soluble in water 1:1200. The phenylmercuric ion is believed to be the active com-

ponent. It differs from other mercurial compounds in that the mercuric ion is the cation. Since bacteria are usually negatively charged, this positive charge on the phenylmercuric ion may be responsible for its high inhibiting power.

Kolmer's bacteriostatic method was used to determine inhibition and with it a modification of the method, used by Birkhaug, in which a very small number of bacteria is used. By Kolmer's method *E. coli* was inhibited by a 1:420,000 concentration. *Staph. aureus* was inhibited by a 1:12,000,000 concentration. Using Birkhaug's method, similar to Kolmer's but using 200 to 2000 bacteria per cc., *E. coli* was inhibited by a 1:2,500,000 concentration. *Staph. aureus* was inhibited by a 1:307,000,000 concentration.

Determining the killing power of the substance, in the case of *Staph. aureus*, offered difficulties because of its high inhibiting power. This was not an apparent factor with *E. coli*. Shippen's modification of the Food and Drug Administration test was found necessary with *Staph. aureus* but even this method, though avoiding the gross error of confusing the inhibiting with the killing power, did not give dependable results. More satisfactory results were obtained by using a given dilution of the disinfectant and varying the time element, using plate counts in addition to the usual test in broth tubes. It is not believed, however, that the true killing concentration of such highly effective mercurials can be determined by any of the usual methods.

G7. Germicidal Properties of Water Containing Minute Amounts of Silver. ROBERT P. MYERS AND J. C. MAUER, Research Laboratories, National Dairy Products Corp., Inc., Baltimore.

In view of the interest in industrial applications of the so-called "oligodynamic" effect of silver in the sterilization of water, the present investigation was undertaken to measure the relative germicidal effectiveness of dilute solutions of silver prepared from: (1) fused silver chloride, (2) silver nitrate, (3) silver acetate, (4) colloidal silver, prepared by passing an arc between 2 silver wire electrodes immersed in distilled water, and (5) a silver solution prepared by the Katadyn process from metallic silver using a dry cell as the source of current.

Two types of water were used in making up the test solutions: (1) sterilized river water having a slightly alkaline reaction, and (2) sterile distilled water to which 10 p.p.m. of $CaCO_3$ had been added and which had a reaction approximately neutral at the time of use.

Escherichia coli and *Staphylococcus aureus* were employed for evaluat-

ing the germicidal potency of the solutions tested. A known number of test organisms were added to the test solutions and plate counts made at various intervals. All tests were made at 20°C.

The results obtained on the 5 types of silver solutions indicate that a concentration of 0.10 to 0.15 p.p.m. of silver ion is sufficient to destroy *Escherichia coli*, when present to the extent of 250,000 per cc., within a period of 2½ hours. *Staphylococcus aureus* appeared to be only slightly more resistant to the action of the silver ion than *Escherichia coli*.

G8. A Study of the Fungicidal Effect of Various Chemicals on Yeast-Like Organisms (Vulvo-Vaginal) and Certain Higher Fungi. H. Close Hesseltine and E. W. Hopkins, University of Chicago, Chicago.

In all, 70 chemical compounds which have recognized fungicidal power or which have been recommended for the treatment of various mycoses were tested for ability to kill representatives of the group of yeast-like organisms producing vulvo-vaginitis.

The technique used was essentially that of a standard loop transfer method. An equal quantity of a broth suspension of young culture of the organisms was mixed with the chemical solution. The exposure time was 5, 15, and 30 minutes after which time a 3 mm. loop of the mixture was transferred to a Petri dish, and agar poured in at once. The chemicals tested were used in concentrations which may be tolerated by the vagina. Under these conditions, some of the chemicals were eliminated from further study.

Since the chemicals would be used in the presence of organic matter and cellular elements, a substitute condition for the vulva and vagina was attempted by tests repeated with the addition of blood serum and red blood cells. Under these conditions, all but a few of the compounds were ineffective. Those showing promise were metallic iodine, mercurophen, mercuric chloride, and chlorothymol.

A number of the compounds tested were known to be fungicides, and yet were very slightly or not at all effective against the yeast-like organisms used under these experimental conditions. The killing power of some of the chemicals employed was tested against members of the higher fungi: a common laboratory contaminant, *Monilia* sp., a plant pathogen, *Fusarium nivale*, and a dermatophyte. Differences were found in the susceptibility of these fungi to various reagents, but a greater number of chemicals were effective against these higher fungi than against the yeast-like organisms.

G9. Germicidal and Detoxifying Properties of Soaps. MILWARD BAYLISS AND H. ORIN HALVORSON, University of Minnesota, Minneapolis.

Pneumococci are especially susceptible to the action of certain unsaturated soaps, such as sodium oleate, linoleate, linolenate, and clupanodonate. These soaps are germicidal at 37°C. in a concentration of 0.005 per cent, using the Reddish technique. Other unsaturated soaps, such as sodium abietate, chaulmoograte, undecylenate, alpha elaeostearate and beta elaeostearates, as well as hydroxylated and saturated soaps, are less effective. Bile salts and sodium salicylate require concentrations of 5 to 10 per cent to kill pneumococci in 10 minutes. Sulfate ester salts are pneumococcidal in concentrations of 0.01 per cent.

Streptococcus lactis is considerably more resistant to the action of soaps than the pneumococcus. In general, soaps which are germicidal against pneumococci are also germicidal against streptococci, but to a lesser degree. There appear to be exceptions to this general rule in the cases of sodium stearate, palmitate, alpha elaeostearate and beta elaeostearate, which are germicidal to pneumococci but not to streptococci.

Escherichia coli and *Staphylococcus aureus* were more resistant than the other two organisms tested. Of the compounds tested, only sodium 3,5-diiodo-salicylate and undecylenate were effective in concentrations of 1 per cent against these two organisms.

Detoxifying tests were made by mixing 1 cc. of a 2 per cent soap solution with an equal volume of normal saline containing 10, 30, 50, 70, or 100 M.L.D. of diphtheria toxin and allowing to stand 15 minutes at 25°C., after which 1 cc. of this mixture was injected subcutaneously into a 300-gram guinea pig, and survival or death of the animal noted. Ability to neutralize diphtheria toxin is a property which is common to all soaps. The degree of their effectiveness varies considerably with chemical constitution. Spatial configuration, as well as functional groups, affects the detoxifying power. One double bond increases markedly the detoxifying ability of a saturated soap, but the addition of further double bonds does not alter it. Hydroxyl groups and triple bonds affect the detoxifying power very slightly. The sodium compounds tested, listed in the descending order of effectiveness, are: chaulmoograte, ricinoleate, oleate, linoleate, linolenate, clupanodonate, alpha elaeostearate, lauryl sulfate, oleyl sulfate, ricinelaidate, beta elaeostearate, ricinstearolate, undecylenate, sativate, stearate, palmitate, myristate, laurate, trihydroxystearate, abietate, 3,5-diiodo-

salicylate, taurocholate, glycocholate, cholate dehydrocholate, salicylate, and gluconate.

The pH of all solutions was accurately controlled by means of the glass electrode.

G10. Participation of Catalase in Cellular Oxidative Processes. T. L. SWENSON AND L. H. JAMES, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Catalase exists in many cells in an inactive or an inhibited condition, and therefore escapes detection except under special circumstances. Observations on a suspension of young cells of *Proteus vulgaris* (Bergey) have shown that the apparent catalase content of the organisms may at times be greatly increased (10 x) by short pre-treatment with a heart muscle extract similar to the antigen of Kolmer.

A connection between catalase and cellular oxidation is shown by measurements on *Proteus* kept for 4 hours at different oxygen tensions. At high oxygen tensions the amount of active catalase is somewhat greater than at low oxygen tensions. The quantity of activable enzyme, however, is very much greater in the oxygenated cells (as determined by the increase in catalase activity after treatment with the muscle-extract activator). It seems probable that the higher rate of aerobic oxidation may stimulate the cells to the production of a potential supply of catalase greater than the actual needs of the organism, as evidenced by the supply of already active enzyme.

The inactive enzyme is distinctly of desmo type, for efforts to separate it from the live cells have failed. With cell death the inactive catalase disappears.

G11. Studies on the Respiratory Mechanism of the Streptococci. MICHAEL A. FARRELL, Department of Bacteriology, Yale University.

A collection of 22 representative strains of the genus *Streptococcus* has been studied with the object of determining the presence of respiratory enzymes, and gaining an insight into the respiratory mechanism of these organisms.

Catalase, cytochrome and indophenol-oxidase could not be demonstrated in the streptococci. The presence of a thermostable peroxidase was established. One hundred and one chemicals, including carbohydrates, amino, fatty, and other organic acids, were tested by Thunberg's technique (with certain necessary modifications) to determine the ability of streptococci to activate them as hydrogen donors. The

streptococci as a group possessed little or no activating power, except toward carbohydrates; most of the hemolytic organisms had no action on any of the sugars or higher alcohols.

The absence of cytochrome and indophenol-oxidase, corroborated by oxygen uptake studies and the ability of these organisms to grow in media containing a high concentration of KCN, indicate that a hemin respiratory mechanism of the Warburg type is absent from the streptococci.

A definite relationship between the thermostable peroxidase and the thermolabile dehydrase of streptococci was shown; this relationship is suggested as constituting an important part of the respiratory mechanism of this group of bacteria.

G12. Variation in Streptococcus hemolyticus. MARTIN H. DAWSON AND MIRIAM OLMSTEAD, College of Physicians and Surgeons, Columbia University, New York.

In a previous report by one of the authors, mucoid, smooth, and rough phases of *Streptococcus hemolyticus* were described. In the present report evidence is presented to show that these variant forms show a close analogy with the corresponding variant forms of pneumococcus and other bacterial species. Various stages in the S → R and R → S transformations are described and on the basis of these observations the suggestion is made that these variations are cyclical in character.

Further studies on the mucoid phase of *Streptotoccus hemolyticus* are reported. These studies show that virulent hemolytic streptococci usually produce mucoid colonies but that organisms in the mucoid phase may or may not be virulent for white mice. The importance of the appropriate culture phases to be employed in subsequent studies with *Streptococcus hemolyticus* and other bacteria is emphasized. Finally, it is pointed out that any attempt to classify the hemolytic streptococci should be based on a study of analogous phases of development.

G13. Dissociation of Saccharomyces aceris-sacchari Fabian and Hall and Pichia alcoholophila Klöcker. L. J. WICKERHAM AND F. W. FABIAN, Michigan State College, East Lansing.

In dissociation studies of yeast *Saccharomyces aceris-sacchari* Fabian and Hall and *Pichia alcoholophila* Klöcker were used. The first named yeast normally produces a bottom growth in malt extract broth and smooth glistening colonies on malt extract agar. When grown and

transferred daily in 0.1 per cent LiCl yeast extract broth, there was a gradual transformation from the normal more or less round cells, 3.5 to 7 microns, into two types of cells. One type of cell was round, 6 to 10 microns, the other elongated, 10 to 16 microns. These cells contained large vacuoles around the periphery of which were located one to 15 or more fat globules, or oil drops. The colonies from both of these types of cells were extremely rough. When cells from these colonies were grown in malt extract broth for one or two days, they produced a rough spreading colony with radial folds which grew over the entire surface of the petri dish in a few days. The cells of this colony were round and measured 6 to 10 microns. When the amount of LiCl was increased to 0.25 per cent and cells from the rough colony were grown in it, extremely long cells, 16 to 22 microns, were produced. The colony produced by these cells in malt extract broth was a rough spreading colony without radial folds. All rough forms returned to the smooth form after several rapid transfers in malt extract broth.

Pichia alcoholophila Klöcker is normally a smooth yeast with growth at the bottom in liquid media. It produces smooth and glistening colonies. The normal smooth cells average about 6 microns in length, are round to slightly elongated in shape, and frequently produce pseudomycelia. A stock culture showing a wrinkled growth typical of rough yeasts was found to contain cells with small oil drops. An attempt was made to dissociate this yeast by growing it in 0.1 per cent LiCl broth, but the yeast could not tolerate it. After a few transfers in yeast extract broth, which has been found in our work to be a mildly dissociating agent, the cells developed large oil drops and vacuoles and the colonies became extremely rugose. Young colonies were composed of cells the same size as the normal smooth cells. Vacuoles were not prominent and oil drops were small or lacking. When the colonies were about six days old, a hanging drop showed many small bodies about 0.7 micron in diameter present along with the cells. The nature of these bodies has not been determined. When the colonies were about 7 or 8 days old, the cells became much larger, averaging 10 microns with vacuoles occupying most of the interior. Metachromatic granules could also be seen moving about inside the vacuoles and the oil drops became very prominent. Pseudomycelial formation was also common at this stage.

When giant colonies of this R form are made on malt extract agar and incubated for 10 to 15 days, secondary smooth colonies appear at several points on the periphery. If these are subplated, they produce

smooth colonies, the cells of which have the same appearance as the normal S cells except for the presence of small oil drops. The oil drops disappear only after many rapid transfers in malt extract broth. The R form when once produced remains stable in yeast extract broth.

G14. Metabolic "Mutation" and Colonial Dissociation in the Genus Bacterium. IVAN C. HALL, University of Colorado School of Medicine and Hospitals, Denver.

The common occurrence of metabolic "mutation" in the genus *Bacterium*, the frequency of *B. coli mutabile* (Massini) in the intestinal tract of man, and complications brought about by *B. coli mutabile* in the recognition of *B. paratyphosus* in the feces of patients and carriers, in food poisoning outbreaks, and in the evaluation of potability of water supplies, are emphasized.

The ultimate criterion of lactose "mutation" is the repeated isolation of rapid lactose fermenters from a parent type which perpetuates both itself and the "mutant" indefinitely, but this criterion is usually preceded by observations of slow fermentation of lactose and of multiple papillae or secondary fermenting colonies on lactose agar media, such, for example, as eosin methylene blue lactose agar. The "mutant" type rarely, if ever when pure, reverts to the parent type, and, as a rule, is indistinguishable from it morphologically, culturally, and serologically, except by its more rapid fermentation of lactose, and by its inability to form characteristic multiple papillae or secondary colonies. Secondary colonies of a different type from those seen in the parent culture on lactose media may occur, however, both in the parent type on non-lactose media, and in the "mutant" on various solid media on long incubation.

The same criteria of metabolic "mutation" are known to apply to certain forms in relation to other carbohydrates and are probably capable of general application.

Data and illustrations are presented to show the simultaneous but independent occurrence of lactose mutation and colonial dissociation in *B. coli mutabile*, *B. cloacae*, *B. dysenteriae dispar*, and *B. dysenteriae* Sonne.

G15. Bacterial Variation: An Inquiry into the Underlying Principles Governing the Cell Morphology of Bacillus megatherium. LEO F. RETTGER AND HAZEL B. GILLESPIE, Yale University, New Haven, Conn.

In the present investigation we have attempted to throw some light on the significance of the variant cell forms so frequently seen in bacterial cultures, by inquiring into the fundamental principles which govern their existence. We have followed, under the microscope, the formation and continued development of *Bacillus megatherium* cells (both variant and ordinary rod forms) growing in microcultures on hanging blocks of agar, and have observed the effect of varying environmental conditions upon cell morphology in this species.

Cell form appears to depend largely upon the environment with which the growing organism has to contend. Slight changes in environment produce striking changes in cell shape. Cells developing on agar which is as yet unaffected by inhibitory metabolic influences occur as "normal" rods, no matter what the age of the culture may be; i.e., the periphery of a 20-day old agar colony is made up of young, hyaline rods, if the colony is far removed from the metabolic influences of surrounding colonies (well-isolated). On the other hand, the peripheral cells of even young colonies vary strikingly on crowded blocks where they are subjected to the diffusing "growth" influences from near neighbors. The type of cell produced seems to depend directly upon the degree of crowding.

Partial oxygen starvation is one of the important factors associated with crowded conditions. That it stimulates at least certain types of cell variation has been clearly demonstrated. When the oxygen supply of a culture is so limited, either by the metabolic influence of associated growth or by mechanical means, as to prevent the development of "normal" rods, globular and streptococcoid forms appear. Such cells are able to withstand conditions of moderately severe anaerobiosis for relatively long periods. They return rapidly to the "normal" rod form when oxygen is again supplied chemically or mechanically.

The factors responsible for variation are definitely unfavorable to growth, and if they accumulate very rapidly in a culture, all development ceases before variation can occur. In other words, variation is possible only where unfavorable and favorable environmental conditions are so balanced as to permit slow growth in the face of adverse circumstances. Variant cell forms do not appear in colonies uniformly distributed throughout the cell mass, but arise in definitely localized areas often in successive rings at colony peripheries. We have observed the growth of clubbed, branched, wedge-shaped, filamentous and globular cells. These variant forms seem to be better able to resist the unfavorable conditions that call them forth than are "normal" rods and, there-

fore, may be thought of as adaptations of the organism which favor the continuation of the species. No evidence has presented itself to us, however, which would indicate that they have any significance as "life cycle" forms.

1G6. The Relation between the Oxidation-Reduction Potential of the Medium and the Dissociation of an Acid-fast Organism. JANET MCCARTER AND E. G. HASTINGS, University of Wisconsin, Madison.

The cultures used were single cell strains of the rough and smooth types of a non-pathogenic acid-fast organism isolated at the University of Wisconsin.

Oxidation-reduction potential measurements, made electrometrically, on dissociating smooth cultures in liquid media, and the dissociation of rough and smooth cultures in oxidized and reduced media, show that oxidized media favor the dissociation of the rough to the smooth type, and reduced media favor the dissociation of the smooth to the rough type.

Plate cultures of dissociating smooth cultures in liquid media were made at intervals. A study of the colonies formed gave evidence that the transition from a smooth to a rough type is not abrupt, but that there are several intermediary types between a very smooth and a very rough type. These various intermediary types dissociate more readily in liquid media than does an extreme rough or an extreme smooth type.

G17. The Use of Semi-solid Agar for the Detection of Bacterial Motility. RALPH P. TITTSLER AND LESLIE A. SANDHOLZER, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

A study of semi-solid agar for detecting motility has been made with 1028 cultures and the results compared with those of the usual hanging-drop technique. The agar (nutrient broth plus 0.5 per cent agar) was inoculated by stabbing and incubated at 37°C. for 6 days. Motility was manifested macroscopically by a diffuse zone of growth spreading from the line of inoculation. Hanging-drop preparations were made from broth cultures incubated at 37°C. for from 12 to 24 hours.

In hanging-drops, 500 cultures were motile. In agar, after 1 day, 497 were motile and 2 were questionable; after 2 days, 520 (including all cultures motile in hanging-drops) were motile and 6 were questionable. Further incubation failed to alter the questionable reactions in

these 6 cultures. Thus, after 1 day, agar yielded results essentially identical with hanging-drops but after 2 days it elicited 4 per cent more positive tests. A few agar cultures, chiefly members of the *Escherichia* genus, after 2 days showed evidence of motility by the formation of nodular outgrowths along the stab. Isolations from these nodules and from the non-diffused stab yielded respectively motile and non-motile strains identical in other cultural characteristics. Thus motile variants have been obtained from some cultures which were non-motile in hanging-drops. The semi-solid agar technique is particularly advantageous in teaching schedules and routine testing, because the results are accumulative and macroscopic.

G18. Studies on the Voges-Proskauer Test. W. Dorner and Esther Hellinger, Swiss Dairy Research Institute, Liebefeld-Bern, Switzerland. (Read by title.)

A comparative study was made on the Voges-Proskauer, Werkman, Leifson and O'Meara methods of detecting the production of acetyl-methyl-carbinol by microörganisms in a dextrose-peptone medium. Likewise the choice of one or more peptones from a range of peptones on the market for the special preparation of the medium was investigated.

In testing the acetyl-methyl-carbinol reaction with organisms of the aerogenes group the choice of a special peptone was not of special consequence. All the methods for conducting the test gave a positive result but certain methods produced a stronger color reaction than others. With other organisms, as for instance *Streptococcus cremoris*, a selection of medium and method of testing was found to be necessary so as not to overlook the possibility of obtaining a positive result.

A peptone broth medium is recommended. This should contain 0.7 per cent dextrose, 1.0 per cent peptone with meat broth as a basis. The following peptones may be used: Peptone Witte, Difco's Bacto-protone and Neo peptone, Fairchild's peptone or Merck's peptone for bacteriological purposes.

A combination of the methods of Werkman and O'Meara proved to give clearer and more positive results than either method alone. The method recommended is to take 1 cc. of the culture in a wide test tube, add 1 drop of 1 per cent creatine, shake; 1 drop of 2 per cent $FeCl_3$, shake well; add 1 cc. 20 per cent NaOH and shake. Place at about 37°C. but refrain from shaking the tube in the early stages of the test so as to observe sooner and more clearly the positive result given by an

eosin-pink to bright red coloration forming at the surface and diffusing downwards through the medium.

G19. The Separation from Veal Infusion of Factors Essential to the Growth of Certain "Fastidious" Bacteria. STEWART A. KOSER AND FELIX SAUNDERS, The University of Chicago, Chicago, Ill.

An attempt was made to separate from ordinary veal infusion the factors which are responsible for growth of some of the more fastidious bacteria. It was found that treatment of an ordinary veal infusion with charcoal resulted in the removal of certain unknown substances necessary for growth of these types and that charcoal was more effective for this purpose than other commonly used adsorbents. These unknown substances, or growth factors, after removal from an infusion in this manner, could be recovered from the charcoal by extraction with hot ethyl alcohol or hot acetone, although it is probable that this recovery was quite incomplete.

These extracts, after evaporation of the alcohol or acetone and subsequent solution of the residue in water, were effective in "activating" an infusion which had been previously treated with charcoal. They were also effective in rendering a standard beef extract-peptone broth more suitable for growth of certain exacting organisms.

The presence of essential growth factors in these extracts could also be shown by addition of the extracts to a synthetic medium. Small amounts of extract were sufficient to permit good growth of a number of organisms which refused to grow in the synthetic medium alone. Thus, several strains of *Corynebacterium diphtheriae*, *Shigella dysenteriae*, *Eberthella typhi* and *Staphylococcus* developed in the synthetic medium when the added material amounted to only 0.0006 to 0.00006 milligram of solids per cubic centimeter of synthetic medium. On the other hand, several strains of hemolytic streptococci, pneumococci, and *Pasteurella* refused to develop in the synthetic medium plus extract.

The growth factors dealt with here are believed to belong in the class of growth accessory substances. Their addition to synthetic media, even though in an impure state, offers a method for securing growth of certain of the more fastidious bacteria and for the study of various phases of metabolism of these types which can best be accomplished through the use of simple culture media. Additional work on other sources of growth promoting principles and on methods for their concentration and purification is now under way.

G20. II. Further Application of Semisolid Media in Cultivation and Identification of Sporulating Anaerobes. ROBB SPALDING SPRAY, Medical School, West Virginia University, Morgantown.

In 1933 the writer presented before the Society an outline of methods adapted to cultivation and identification of the sporulating anaerobes, which obviate all of the difficult and specialized technic commonly applied to this group of organisms. Since that time these principles have been extended over a larger number of species.

The tentative key, previously presented, has also been enlarged to cover these additional organisms. Many of these descriptions are based upon study of but a single strain, but such strains are of authentic origin. Others are based on study of from 5 to 25 strains. All tests have been repeated at least 3 times, and are regarded as reliable under conditions afforded by these methods.

In view of the fact that many descriptions are based upon study of such single strains, the key is still to be regarded as tentative only. We believe, however, that the significant reactions will be substantiated by further extension of the work. Results of further studies will be presented in subsequent reports.

G21. Stimulation of Bacterial Growth Rate by Methyl Germanic Oxide. PHILIP L. CARPENTER, MACDONALD FULTON AND C. A. STUART, Biological Laboratory, Brown University.

Methyl germanic oxide, 0.01 molar, in nutrient broth was found to have a stimulating effect upon the growth rate of organisms of many genera. Growth rate constants indicated that stimulation was greatest during the early logarithmic phase, but that the growth rate fell to zero earlier in stimulated cultures than in controls. A tendency to prolong lag was also observed. The action of methyl germanic oxide resembled in many respects the stimulating action of cations studied by Winslow and coworkers.

G22. Growth Curves of Escherichia coli and Salmonella schottmuelleri in Mixed Cultures. MACDONALD FULTON AND C. A. STUART, Biological Laboratory, Brown University.

The growth curves of pure and mixed cultures of *Es. coli* and *Sa. schottmuelleri* were studied in a synthetic medium. The chief difference between growth curves in the mixture and in pure cultures was in the length of the lag phase. Whichever species was numerically inferior at the outset underwent a prolonged lag in mixed culture, beginning

normal growth only after the other species had reached the period of declining numbers. Other differences between the growth curves of pure and mixed cultures were apparently due to pH changes in the medium. The reported antagonistic action of coli against schottmuelleri may be attributed to prolongation of the lag of the latter, not to a bactericidal effect.

G23. Some Quantitative Relationships in Bacterial Population Cycles. M. W. JENNISON AND J. W. M. BUNKER, Massachusetts Institute of Technology, Cambridge.

The growth cycles of 4 common saprophytes—*Escherichia coli* (2 strains), *Aerobacter aerogenes*, *Serratia marcescens*, and *Chromobacterium violaceum*—were determined in nutrient broth at 22°, 27°, 32° and 37°C., the last temperature being the optimum for rate of reproduction of the various organisms. To insure a maximum rate and longer period of reproduction the inoculum was small (1000 to 2000 cells per milliliter) in every case. Enumeration of cells was by the plate count, using 5 plates of a suitable dilution of pooled, duplicate cultures. Counts were made every 2 hours for 48 hours.

At any one temperature, the rate of multiplication during the logarithmic phase of growth was nearly the same for the different organisms. An average generation time of 60 minutes at 22°, and 22 minutes at 37°C., was found. Calculation of the temperature quotient Q_{10}° for various temperature intervals showed that it decreased continuously with increase in temperature; that is, as the optimum was approached the increase in temperature had relatively less and less effect upon the rate of reproduction.

While the maximum *rates* of reproduction attained by the different organisms at the same temperature were nearly the same, the maximum *numbers* of cells finally reached in the cultures by different species at the same temperature showed wide variation; but for any one species the maxima reached were fairly consistent irrespective of temperature.

A constant relationship was found between the *length* of the logarithmic growth phase at any temperature and the *rate* of multiplication during that phase. At each of the temperatures used, the ratio of rate of reproduction (as generation time in minutes) to length of the logarithmic growth phase (in hours) was quite consistently 5:1.

Considered as typical population curves, these bacterial growth cycles were, as a rule, very asymmetric. While a "normal" S-shaped population curve is considered to be, theoretically at least, symmetrical, these

curves all showed varying degrees of asymmetry in the upper part of the "S", which asymmetry was outside of the experimental errors of technique.

G24. Chitinovorous Bacteria,—a Preliminary Survey. ANNE G. BENTON, Vassar College.

By use of suitable enrichment and plating media, over 300 apparently pure cultures of chitinovorous bacteria have been isolated from a variety of sources, including water, soil, decaying animal matter and the alimentary tracts of birds, bats, frogs and fishes. At least 18 distinctly different types can be distinguished. Some of them seem to be fairly widespread in nature; others were isolated from but one source. None correspond to species now listed in Bergey's Manual, though a few may be related to recognized species. Atrichic, monotrichic and peritrichic rods are represented, as well as vibrios, spindle-shaped cells and pleomorphic forms. Among the latter types, a few seem to resemble cellulose bacteria from the soil, described by Winogradsky and others. The various morphological types exhibit interesting fermentative peculiarities, and are alike only in their ability to attack chitin. Some exhibit characteristic pigmentation. Their striking dissimilarity in gross and microscopic morphology, their differences in ability to attack various substrates, and their different types of fermentative attack on available substances, make it obvious that they should not be regarded as belonging to a single chitinovorous genus, but rather that they should be included in genera now recognized (or later to be defined) by morphological as well as cultural characteristics.

G25. The Fermentation of Cellobiose by Bacteria. RALPH P. TITTSLER AND LESLIE A. SANDHOLZER, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

The ability to ferment cellobiose has been determined for 755 cultures, representing approximately 50 species within the following genera: *Aerobacter, Escherichia, Proteus, Salmonella, Eberthella, Shigella, Alcaligenes, Serratia, Chromobacterium, Flavobacterium, Pseudomonas, Bacillus, Staphylococcus, Micrococcus* and *Sarcina.*

The results may be briefly stated as follows: acid and gas were produced by the *Aerobacter* cultures and most of the *Escherichia-Aerobacter* "intermediates," but not by typical members of the *Escherichia* group. All strains of *Eb. typhi, Sal. aertrycke* and *Sal. schottmuelleri* and 17 of 19 strains of *Sal. enteritidis* attacked this carbohydrate after from 4 to 10

days, whereas all strains of *Sal. suipestifer*, *Sal. paratyphi*, *Sal. gallinarum*, *Sal. pullorum*, *Sal. morgani* and the dysentery bacilli failed to do so within 14 days. Likewise, there were differences between certain other species, particularly those of the *Proteus* and *Bacillus* genera. In general, the cocci failed to ferment cellobiose. Since certain closely related groups or species differ in their ability to ferment cellobiose, this characteristic can serve a useful purpose in bacterial taxonomy.

G26. Studies on the Micrometabolism of Yeast Cells of Different Species. II. The Influence of KCl and $CaCl_2$ on the Glucose Esterification. ERNEST A. PRIBRAM AND LOUIS KOTLER, Department of Bacteriology and Preventive Medicine, Loyola University, Chicago.

In the first paper (J. Bact., **27**: 24, 1934), differences have been reported between a beer yeast and a milk yeast concerning their capacity to combine with different carbohydrates, dextrose, galactose and lactose. Our present problem is to determine the influence of potassium and calcium chloride on the glucose combining power of yeast cells of different origin. The types of yeast cells were: *Saccharomyces cerevisiae* Hansen, type Frohberg, sedimentary beer yeast ("Frohberg"), "*Saccharomyces*" *kephir* Beijerinck, from kefir milk (the correct scientific name should be: *Torulopsis beijerincki*), *Torulopsis rosea*, isolated from air, and *Cryptococcus hominis* Busse, isolated from a case of thrush. The methods used for the determination of the capacity of yeast to combine with dextrose, were those published in the previous article. To 1 cc. of the yeast suspension of 1000 cells per cubic millimeter, 0.02 cc. of 0.1 N solutions of the salts was added. All cultures were three days old, grown on dextrose agar. The combining power of the yeast for dextrose is based on its high content of phosphates (50–60 per cent of the inorganic ash), as stated by Wertheim, who found, that after adding glucose no glucose reaction (aldehyde reaction) was present in the thoroughly washed cells, unless the phosphorus-glucose ester had been hydrolysed by sulfuric acid. The results were as follows: The electrolytes KCl and $CaCl_2$ were in all experiments antagonistic in their action on glucose esterification by yeast cells. Their effect, however, was the opposite with different types of yeast. KCl decreased and $CaCl_2$ increased the esterification (loss of glucose in the suspension), if added to Frohberg yeast or to *Torulopsis*. KCl increased and $CaCl_2$ decreased the esterification, if added to kefir yeast or to *Cryptococcus*. It seems to be very likely that a certain ratio of different electrolytes,

especially of potassium to calcium ions is favorable for the esterification of glucose by yeast cells.

Analyses of the ash of Frohberg yeast and of a yeast from animal tissue (thrush) as found in the literature showed that the Frohberg yeast contained a high percentage of potassium, and a low one of calcium. The ratio is: K:Ca = 28:1 or, at least, 10:1. The yeast isolated from animal tissue had a relatively low percentage of potassium and a high one of calcium. The ratio is K:Ca = 9:14 or 1:1.55. This may explain why adding potassium chloride counteracts the reaction between glucose and phosphates, why calcium favors it when Frohberg's yeast is used, and why the opposite holds true for yeasts isolated from and adapted to animal tissue. Further results with other electrolytes will be published later.

G27. Slow Lactose Fermenting Bacteria Pathogenic for Young Chicks.
KEITH H. LEWIS AND E. R. HITCHNER, Department of Bacteriology, University of Maine, Orono.

A study has been made of the characteristics of a microörganism, isolated from several lots of infected chicks, which exhibited symptoms simulating pullorum disease. The delayed fermentation of lactose served to differentiate it from *S. pullora* and the other recognized fowl pathogens. The organisms are pathogenic for guinea pigs and young chicks and in the latter produce symptoms and pathological changes which resemble those in pullorum disease.

The cultural and biochemical tests were made according to the methods advocated in the Manual of Methods for Pure Culture Study.

These bacteria are motile, Gram negative, non-capsulated, non-spore forming, facultative, short rods; grow well on ordinary nutrient media with characteristics closely resembling those of *E. coli*; curdle milk only after 20 to 30 days; reduce nitrates; form hydrogen-sulphide from peptone; do not liquefy gelatin; produce no indol; grow well in Koser's citrate medium; do not form acetyl-methyl-carbinol; methyl red positive.

Acid and gas are produced in 24 hours from arabinose, xylose, dextrose, levulose, galactose, maltose, trehalose, mannitol and sorbitol. Neither acid nor gas is formed from sucrose, cellobiose, raffinose, salicin, glycogen, dulcitol, adonitol and inositol. The appearance of acid and gas in lactose broth is delayed for 2 to 5 days. By aging the organisms for two weeks in lactose broth they acquire the ability to ferment this sugar in 24 hours. The aged organisms curdle milk more rapidly but in all other respects are identical with the original strains.

On Endo agar, purple lactose agar and Russell's double sugar agar the bacteria show characters which resemble those of the paratyphoid group. The organisms exhibit a remarkable tolerance toward Brilliant Green in that they grow readily on veal infusion agar in the presence of 1:25000 concentration of the dye.

Agglutination tests show little antigenic relationship of these organisms to *E. coli*, *A. aerogenes*, *S. pullora*, *S. anatum*, *S. aertryke*, *S. enteritidis*, *S. schottmuelleri* or *Proteus vulgaris*. Slow lactose fermenting bacteria, obtained from other workers, which show an apparent close antigenic relationship, have been distinguished from the avian strains by agglutination-absorption.

The bacteria exhibit characteristics which fail to fit well into any of the established genera of the **Bacteriaceae** and certainly are not identical with any of the recognized species. Because of the peculiar combination of characteristics displayed by this organism and the unsatisfactory state of bacterial nomenclature with respect to the slow lactose fermenting organisms no specific name is suggested.

G28. The Relative Productivity of Various Fermentation Media as Compared with Standard Lactose Broth by the Butterfield-Hoskins Method. R. E. NOBLE AND JOHN L. WHITE, Board of Health, Chicago, Ill.

Fuchsin lactose, formate ricinoleate, crystal violet, brilliant green lactose bile and Difco lactose broths were each tested separately, in parallel with standard lactose broth, pH 6.8–7.0, as routinely prepared in the Chicago Board of Health Laboratories. Two methods of testing were used: (1) Conventional inoculation from 61 coli-aerogenes stock strains, and (2) the Butterfield method, with results expressed by the Hoskins Most Probable Number, using a single stock strain of *Bact. coli* as the test organism.

Uniformly all trial and standard media produced gas within 42 hours of incubation at 37°C. after the conventional inoculation of the 61 stock strains, in mass amounts. On this basis alone all the trial media would be approved for further study of their practical usefulness. The results by the Butterfield-Hoskins method, on the other hand, were quite different. This method is a quantitative one using a *limited number* of organisms for testing, and hence is more sensitive for the evaluation of media.

In order to compare the productivity of each trial medium with the standard medium impartially, proper allowance had to be made for a

normal variability in readings in any medium. To this end, the normal variation of standard lactose broth readings was determined. The mean percentage, minus deviation, 90.6, was arbitrarily taken as a minimum lower limit of variability to be met by more than half of the results with any given trial medium, in three or more successive tests. The most probable number for the trial medium divided by the corresponding value for the standard medium, gave the percentage value for comparison of each with the standard. The following trial medium values were obtained: fuchsin lactose broth: 33.8, 30.4, 14.5; formate ricinoleate broth: 95.9, 98.6, 90.1; crystal violet broth: 103.3, 113.5, 80.7, 71.6, 47.5; brilliant green lactose bile: 89.7, 108.7, 66.9, 82.3; Difco lactose broth: 114.4, 108.1, 100.5. Of these media only formate ricinoleate broth and Difco lactose broth met requirements for further study in these tests.

Each comparison required 50 tubes of the trial medium and 50 tubes of standard medium in each of three dilutions, or 300 tubes in all. The above data, therefore, represent observations on 5400 fermentation tubes. Similarly, 3600 tubes of standard medium were used in determining the range of normal variation of the readings.

G29. Effect of Temperature and Boric Acid on Gas Production in the Colon Group. REESE VAUGHN AND MAX LEVINE, Department of Bacteriology, Iowa State College, Ames.

A medium containing boric acid is presented which may be useful for the detection of the genus *Escherichia* and to inhibit growth of the genera *Aerobacter* and *Citrobacter*. The medium has the following composition: Proteose peptone (Difco), 10 grams; lactose, 5 grams; K_2HPO_4 (anhydrous), 12.203 grams; KH_2PO_4, 4.085 grams; boric acid, 3.25 grams; Andrade's indicator, 10 cc.; and distilled water, 1000 cc.

The medium was sterilized at 15 pounds pressure for 15 minutes. The inoculum consisted of a 2.5 mm. loop of 24-hour nutrient broth culture of the test organism.

Of 148 strains of *Escherichia* studied 98 per cent grew luxuriantly in this medium after 48 hours at 43° to 44°C., whereas of 181 strains of the genus *Aerobacter* observed, only 3.3 per cent showed growth. Of 53 strains of *Citrobacter* studied there was no evidence of growth for any of the organisms.

There was some evidence of inhibition of gas production with some strains where growth did occur. Of the *Escherichia* cultures studied 95.3 per cent produced gas whereas not a single strain of the 181 *Aero-*

bacter or 53 *Citrobacter* cultures produced gas after 48 hours at 43–44°C. (temperature of the medium).

G30. A Glucose Iron Bismuth Sulphite Medium for Quantitative Estimation of E. typhi in Water, Sewage and Sewage Sludges. C. C. RUCHHOFT, Laboratories, Sanitary District of Chicago.

In work undertaken by the Sanitary District of Chicago a medium for isolation and quantitative estimation of *E. typhi* in sludge and sewage was needed. It was found that the older isolation media (Endo, E.M.B. and brilliant green agar) were too inhibitive of *E. typhi* when compared with nutrient agar and not sufficiently inhibitive of other sewage organisms. Wilson and Blairs' medium was found much too inhibitive of *E. typhi*, it being only 1.7 per cent as productive as nutrient agar. After many experiments with numerous modifications of the Wilson and Blair medium a satisfactory medium was found. This medium contains a lower concentration of the sugar, buffer, and all the inhibitive salts of the latest Wilson and Blair medium and contains a small amount of ferric chloride in addition. The iron salt is necessary to produce the characteristic *E. typhi* colony in the medium of lower concentration. This medium is 92 per cent productive of *E. typhi* and only 1.8 per cent productive of other sludge bacteria when compared to nutrient agar.

The medium base agar is prepared as follows: to 1000 cc. distilled water add peptone, 5 grams; beef extract, 3 grams; glucose, 3 grams; $Na_2HPO_4 \cdot 12H_2O$, 2 grams; washed agar, 20 grams. Boil until solution is complete; bottle in 150 cc. amounts (without clarification) and sterilize at 15 lbs. for 15 minutes. pH adjustment is unnecessary.

The following stock solutions are prepared to be used with the medium:

(1) Bismuth citrate, 3 per cent solution. This is made by boiling 30 grams of bismuth citrate (U. S. P.-Merck) in 1000 cc. of water and adding aqua ammonia (sp. gr. 0.90) until solution is complete. About 30 cc. of ammonia is required. (2) A 20 per cent Na_2SO_3 solution. (3) A 1.0 per cent ferric chloride solution, 2 per cent if $FeCl_3 \cdot 6H_2O$ is used. (4) A 1.0 per cent brilliant green solution.

For convenience the above solutions are added from two "mixes" as follows: Mix A consists of 36 cc. of solution (1) and 38 cc. of solution (2) and 26 cc. of sterile distilled water. Mix B is prepared by adding 12 cc. of solution (4) to 100 cc. of solution (3).

When the base agar is melted, mix A is shaken and 10 cc. is added per

150 cc. of agar. This is followed by 3 cc. of mix B. The medium should then be cooled to 45°C. and added in 10 cc. amounts with a pipette to the plates containing the samples under examination, care being taken to keep all the precipitate in suspension during the distribution of the medium.

Plates poured with the medium are incubated for 20 to 24 hours at 37°C. *E. typhi* colonies are small with a typical delicate brown halo. Certain other sulphite reducers produce larger colonies with a slightly darker brown halo. Suspicious colonies are counted and fished to Russell's double sugar medium slants and are further confirmed by other fermentation tests and agglutination.

E. typhi has been isolated from Chicago activated sludge with this medium. Death rate curves of *E. typhi* in stored activated sludge have been obtained. *E. typhi* has been isolated from sludge samples with this medium when for each *E. typhi* present there were 1000 other members of the coli-aerogenes group and 25,000 other bacteria.

G31. Bacterial Contents of Chlorinated Swimming Pools During Periods of Use. W. L. MALLMANN, Department of Bacteriology, Michigan State College, East Lansing.

The use of the sodium thiosulphate treated sample bottle in collecting samples of water from chlorinated swimming pools has demonstrated that during periods of heavy bathing loads, large numbers of bacteria are present, as revealed by 37°C. total counts, *Escherichia coli* indices and streptococci indices. Data are presented to show the effect of varying bathing loads at different chlorine residuals in different sized pools. By regulating the bathing load, the bacterial contents of the pools could be held within the present standards recommended by the American Public Health Association Committee on Bathing Places. A minimum of 0.2 p.p.m. available chlorine was found to be low. In general, the chlorine residual should be maintained at 0.4 p.p.m. The critical period of pollution was found to occur shortly after the bathers entered the water.

G32. Formate Ricinoleate Broth—a New Medium for the Detection of Colon Organisms in Water and Milk. C. N. STARK AND C. W. ENGLAND, Cornell University, Ithaca, N. Y.

A new medium, consisting of: 0.5 per cent peptone, 0.5 per cent lactose, 0.5 per cent sodium formate and 0.1 per cent sodium ricinoleate, has been used for the detection of colon organisms in water and milk.

This medium inhibited the growth of all of those organisms, tested by us, which have been mentioned by other workers as being responsible for "false tests." The medium accelerated the growth of the bacteria belonging to the *Escherichia-Aerobacter* group, caused the total population of bacteria to be much larger, and very markedly increased the total amount of gas produced.

The sodium ricinoleate inhibited the growth of the bacteria which may cause positive presumptive tests which can not be confirmed. Yet the results of our experiments indicated that when one colon organism is present growth will occur and large amounts of gas will be produced.

Sodium formate accelerated growth and caused the total number of bacteria to be larger, partly because of its buffering effect. The pH of the medium after two days' incubation was above 6.0. The amount of gas produced was increased because bacteria belonging to the *Escherichia-Aerobacter* and *Salmonella* groups can produce gas from formic acid, in the absence of any other fermentable substance. Gas production from the formate resulted in the accumulation of NaOH and $NaHCO_3$, which substances maintained the high pH in the medium.

The addition of the amounts of protein material which would of necessity be added to the medium in testing water or milk for the presence of colon organisms, did not materially change the surface tension of the medium.

Formate ricinoleate broth has been used very satisfactorily in testing several hundred samples of water and milk.

G33. A Critical Study of Some Media Used for the Detection of Colon Organisms in Water and Milk. C. N. STARK AND L. R. CURTIS, Cornell University, Ithaca, N. Y.

Standard lactose broth has been criticized because it permits the growth of gas-producing organisms of no known sanitary significance. Media, containing different dyes in various amounts, have been suggested to replace lactose broth.

Salle's crystal violet broth, Dominick and Lauter's broth, gentian violet bile broth and brilliant green bile (2 per cent) broth have been studied by us to determine: (1) the growth inhibiting and growth accelerating substances in these complete media and their various constituents; (2) the ability of small numbers of colon organisms to initiate growth in the media; and (3) the detoxifying action caused by adding to tubes of the broths the amounts of milk (0.1 and 1.0 cc.) which would be added to the media when they are used in the detection of colon organisms in milk.

Crystal violet, methylene blue, gentian violet and brilliant green were shown to be the growth inhibiting substances in the media. Bile was found to decrease the toxic action of the dyes used and to accelerate the growth of "false test organisms" in media in which oxgall was used.

Small numbers of colon organisms were able to initiate growth and produce gas in brilliant green bile broth. Small numbers of these organisms did not grow and produce gas in Salle's crystal violet broth.

Many bacteria responsible for "false tests" were able to grow in these media. The addition of 1 cc. of sterile milk to tubes of the media materially increased the number of "false test organisms" which were able to grow. Failure to inhibit the growth of these gas-producing bacteria, not known to be of any public health significance, is a major weakness in the media.

G34. Eosin Methylene Blue Smear Agar for Rapid Direct Count of E. coli. H. W. Gehm and H. Heukelekian, New Jersey Agricultural Experiment Station, New Brunswick.

The standard eosine methylene blue agar for the confirmation of *E. coli* has been used for the direct count.

The obvious difficulties involved in using this medium for direct count by the pour method have led us to employ a smear method which gives more characteristic surface growths. The various dilutions are smeared on the hardened surface of this medium, allowed to dry and incubated for 24 hours.

After 24 hours incubation characteristic colonies are counted. Good distribution was observed in all cases, and very few other organisms developed. The counts obtained in 24 hours were consistently higher than those by the 48-hour brilliant green tube method but proportional to them. Incubation for an additional 24 hours gave few additional *E. coli* colonies on the plates.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

M1. The Place of Bacterial Allergy in the Immunization Process. L. Dienes, Massachusetts General Hospital, Boston.

The immunization process does not start with the production of antibodies and their diffusion in the tissues and body fluids, but with a period of pure tissue hypersensitiveness the development of which precedes the production of circulating antibodies a few to many days. Guinea pigs after injection of a few milligrams of egg white give a slight

but definitely positive skin reaction as soon as the fourth day after injection, while the anaphylactic sensitiveness and antibodies do not appear usually before the eighth day. Similar observations were made on rabbits and on man. These slight early reactions differ considerably from the later reactions (a few weeks after treatment) and from the reactions of passively sensitized animals. The early reactions are delayed and permanent; they are similar in every respect to slight tuberculin reactions, in contrast to the quickly developing wheal characteristic of passively sensitized animals. If tuberculous guinea pigs are sensitized with egg white, under appropriate conditions, at the end of the first week the slight early reactions are replaced by strong necrotic reactions corresponding in every respect to strong tuberculin skin reactions. Histological studies furnish further evidence for the connection between the early slight reactions and bacterial allergy. For the slight tuberculin reaction and the early reactions, a slowly developing but eventually intensive cellular infiltration is characteristic, in which mononuclear cells predominate. The skin reactions of passively sensitized animals are markedly different, being characterized by quick exudation of serum and polymorphonuclear cells.

Bacterial allergy is the strong development of the early phase of the specific response to antigen preceding the production of circulating antibodies. Every active immunization process passes through this phase, but its development is strongly influenced by certain infectious diseases. The influence of these diseases was proven by treating animals with indifferent antigens such as egg white or horse serum during the disease, and it was also observed that the anatomical lesions play an important rôle in this effect. In a tuberculous guinea pig the most effective method to produce a strong tuberculin type of sensitiveness is to inject the egg white into the tuberculous lesions.

To form an opinion of the rôle which allergy plays in the immunity and the healing of infectious diseases, it is important to keep in mind that the allergy produces mainly a cellular reaction in the tissues which come in contact with the antigen, consisting mostly of mononuclear cells. In those diseases in which allergy persists, as in tuberculosis, the defensive reaction is mainly a tissue reaction. Once antibodies are formed, the reaction in the tissues to the antigen is a quick exudation. The influence of allergy on the tissue reaction is probably present also in acute diseases. But the allergy probably serves a more general purpose. The inefficacy of serum therapy in most diseases makes it very probable that the active immunization of the tissues, and not the diffusion of

antibodies, is the most powerful factor in the healing, and the bacterial allergy is a manifestation of the early phase of the active immunization process.

The immunity response during the disease differs in many respects from the response observed in normal animals after the introduction of simple antigens.

M2. Duration of Local Skin Reactivity Induced by Bacterial Filtrates.
GREGORY SHWARTZMAN, Laboratories of Mount Sinai Hospital, New York, N. Y.

It was reported in early publications that the state of local skin reactivity to bacterial filtrates disappears within 48 hours following the intradermal injection of preparatory bacterial factors. Subsequent experiments demonstrate the following:

The duration of reactivity depends upon the mode of preparation of filtrates and the microorganisms employed. Thus, it lasts for 96 hours with meningococcus "agar washings" filtrates; for 72 hours with *B. typhosus* "agar washings" filtrates; for 48 hours with *B. typhosus* broth culture filtrates, and only for 24 hours with *B. typhosus* "agar washings" filtrates previously heated in the Arnold sterilizer for 20 minutes. Comparative titrations of these preparations show that the duration of vulnerability is in direct relationship to the strengths of the preparations employed.

It was also previously reported that the skin sites prepared by bacterial filtrates undergo severe hemorrhagic necrosis when acted upon by toxic principles resulting from intravascular interaction of non-bacterial antigens with homologous antibodies. This interaction can be obtained either by separate intravenous injections of the antigen and the antibody, or by intravenous injections of an antigen into rabbits possessing active acquired homologous antibodies. It was of interest to study the duration of local skin reactivity induced by bacterial filtrates to these antigen + antibody complexes. The duration of vulnerability was studied weekly in rabbits sensitized by one to 4 repeated weekly injections of horse serum. The state of reactivity disappeared uniformly in sensitized rabbits, 96 hours after the intradermal injections of a meningococcus "agar washings" filtrate.

In dilution, the skin-preparatory potency of *B. typhosus* "agar washings" was titrated in normal and horse serum-sensitized rabbits. Rabbits received intradermally 0.25 cc. of dilutions ranging between 1:50 to 1:300 and 24 hours later intravenously 1 cc. per kilo of body weight

of dilution 1:100. The skin-preparatory titer was the same in normal and sensitized rabbits (800 to 1000 units per 1 cc.).

It is definitely shown, therefore, that the duration of the local skin reactivity elicited by bacterial filtrates is, first, in direct relationship to the potency of these filtrates and that second, it has no apparent bearing upon the anaphylactic sensitization (i.e. Arthus phenomenon) to animal proteins.

M3. The Relationship of the Potency and the Antigenicity of the Tuberculin Protein to its Molecular Size. FLORENCE B. SEIBERT, The Henry Phipps Institute of the University of Pennsylvania, Philadelphia.

Various protein fractions obtained from tuberculin differing widely in their physico-chemical properties, such as precipitability, solubility, filtrability, heat coagulability, etc., are nevertheless equally potent in their ability to elicit the specific tuberculin skin reaction. On the other hand, the antigenicities, i.e., the ability to elicit precipitins and the Arthus reaction in normal animals by repeated injections of these antigens, are quite different. For example, the fraction made by ammonium sulfate precipitation is an excellent antigen, better, in fact, than the well-known crystalline egg albumin, serum proteins, etc., while fractions isolated from OT or made by heating an antigenic fraction of tuberculo-protein with weak alkali are completely lacking in antigenicity. Molecular weight determinations by means of osmotic pressure measurements show in these fractions variations as great as 25,000 to 2,000. Moreover, there is a close parallelism between the size of molecule and its antigenicity. In other words, the antigenicity depends upon the size of the molecule, whereas the specific tuberculin skin potency is independent of the size of the molecule, and appears to reside in a small unit of the whole molecular complex.

Just as it has been possible to abolish antigenicity by disaggregating the large into the small molecules without loss in specific skin potency, so it should be possible again to build up the large antigenic molecule from the small non-antigenic pieces. In view of the fact that antigenic properties of toxins can be improved by adsorption to inert colloids, as shown by Glenny and coworkers and many other investigators, and that the precipitinogen properties of antigens in general were increased by Hektoen and Welker through adsorption to aluminium hydroxide, a study of the effect of adsorbing the small molecular tuberculin molecule to aluminium hydroxide was made. It was found that such adsorption

(i.e., to aluminium hydroxide or to charcoal) rendered the previously non-antigenic fraction of molecular weight of about 4,000 capable of eliciting in normal rabbits as high a precipitin titer as the large antigenic molecule of molecular weight 25,000 and also increased its ability to provoke the Arthus reaction. Moreover, some degree of inhibition in the *in vitro* precipitin reaction could be obtained by means of the small non-antigenic molecule. These results strongly suggest a similarity between the small molecular specifically potent molecule (as probably exists in OT) and haptens in the sense of the word as used by Landsteiner. That is, it is practically unable by itself to elicit antibodies, but is capable of acting specifically upon antibodies when they are present, as in the normal sensitized or in the tuberculous animal.

M4. The Relation of Antibody Content to Allergy and Resistance in Animals Vaccinated with B.C.G. B. J. CLAWSON, Department of Pathology, University of Minnesota.

Rabbits were vaccinated by injecting them with from 1 to 2 mgm. of B.C.G. four times at weekly intervals. Some animals were injected with living organisms and others with heat-killed organisms. Injections were given subcutaneously in some animals and intravenously in others with living and heat-killed organisms, respectively.

The degree of allergy, if present, was determined by the Mantoux test. The amount of resistance developed was observed by comparing the length of life and the degree of tuberculosis, if present, in vaccinated and normal animals infected with a virulent bovine strain of the tubercle bacillus.

The concentration of the antibodies in the blood was estimated by the complement fixation, agglutination and opsonic index tests.

The experiments showed a correlation between the antibody content and resistance, but there was no definite proportionate or necessary relation between the presence of allergy and antibodies.

M5. Tissue Extracts and Anaphylactic Shock. L. GERBER AND E. GERWE, The Wm. S. Merrell Co., Cincinnati, Ohio.

When saline extracts of lung, muscle, kidney or other tissues are injected into the veins of a rabbit or guinea pig, a series of symptoms arises that so markedly simulates true anaphylactic shock that some confusion still persists concerning the two phenomena. Differentiation of death from thrombosis produced by tissue extracts and that from acute anaphylactic shock may be effected by (1) the use of a zoologically

unrelated species for sensitization; tissue extracts do not exert a coagulant effect *in vivo* on unrelated species, (2) the presence of dark red, organized blood clots in the right heart of all animals that have been killed by tissue extracts, (3) the constancy of the characteristic lesion of acute anaphylactic shock in the guinea pig; completely emphysematous lungs that appear rarely with thrombosis produced by tissue extracts, and (4) a dissimilarity in the syndrome when pigeons are employed as experimental subjects.

M6. Loewenstein's Method of Culturing B. tuberculosis. L. M. KOPELOFF, N. KOPELOFF, L. E. HINSIE, AND J. L. ETCHELLS, Department of Bacteriology, Psychiatric Institute and Hospital, New York.

Because Loewenstein has claimed that *B. tuberculosis* may be cultured by his methods from 40 per cent of dementia praecox patients we arranged to send him samples from such patients while attempting to culture duplicate samples in our own laboratory.

In most instances triplicate samples of blood from patients with mental and physical disease as well as from control subjects were sent to Loewenstein as individual specimens of unknown origin. Among 60 controls 5 positive macroscopic cultures of *B. tuberculosis* were reported as coming from healthy young males in whom there had been no demonstrable tuberculous infection. No macroscopic cultures of *B. tuberculosis* occurred in 25 cases of active pulmonary tuberculosis or 24 cases of eye infection (2 of which were diagnosed tuberculous retinitis). Twenty-seven positives were reported in 96 cases of dementia praecox and 7 positives in 19 miscellaneous psychoses and neuroses.

The above data contradict Loewenstein's previous claims of: (*a*) a high incidence of positive blood cultures of *B. tuberculosis* in febrile pulmonary tuberculosis and (*b*) in dementia praecox; and (*c*) a complete absence of positive findings in controls.

In our own laboratory by rigidly following Loewenstein's method on 375 blood samples from the same patients we were unable in any instance to culture *B. tuberculosis*.

Of 8 spinal fluid specimens from suspected cases of tuberculous meningitis 6 yielded positive cultures of *B. tuberculosis* on Loewenstein medium in 16, 23, 27, 45, 52 and 125 days, respectively.

Loewenstein's results reported here and our failure to obtain any positive blood cultures indicate that there is no justification for our further study of patients with dementia praecox by his methods.

M7. The Rôle of Mucin in the Production of Experimental Lobar Pneumonia in the Rat. W. J. NUNGESTER AND L. F. JOURDONAIS, Department of Bacteriology, Northwestern University Medical School.

A chance observation led to the finding that the virulence of bacteria could be greatly increased if the organisms were suspended in sterilized gastric mucin for inoculation. The possibility that mucin might be a factor in spontaneous disease was suggested by this finding, and the rôle of mucin in experimental pneumonia has been investigated with the following results. Forty-nine white rats were inoculated intratracheally with 0.00005 or 0.000005 cc. of a 24-hour glucose broth culture of a type III pneumococcus suspended in 0.5 cc. of mucin. Forty-three of these died, of which 32 had an entire lobe consolidated, 9 had over 50 per cent of a lobe consolidated and two had less than 50 per cent of a lobe consolidated. The lesions resembled grossly and histologically the various stages of lobar pneumonia except the stage of resolution. Fibrinous pleurisy and pericarditis were commonly seen.

Similar doses of the organism suspended in saline were inoculated intratracheally into 35 rats. Three of these died. Consolidation was noted in these and in 3 others that were sacrificed. Of 31 rats inoculated with mucin alone 1 died. A mild reaction to the mucin inoculation was noted in 13 of the 30 animals sacrificed.

The foregoing results indicate that mucin in the respiratory tract may play an important rôle in the pathogenesis of lobar pneumonia. It is believed that the mucin acts in the same way as the starch used by Terrell and Robertson in their study of experimental pneumonia in the dog and that in both instances there is mechanical interference with the normal defense agents of the body, probably the phagocytes.

M8. The Virulence of H. pertussis. JOHN A. TOOMEY, City Hospital and Department of Pediatrics, Western Reserve University, Cleveland, Ohio.

When the minimal lethal dose of freshly isolated hemolytic *H. pertussis* organism is ascertained for the guinea pig and then comparative weights of wet, freshly isolated, non-hemolytic organisms are injected intraperitoneally in other animals, it is found that the non-hemolytic organisms are as virulent as the hemolytic ones. *H. pertussis* cultures decrease in virulence after they become acclimated to veal brain, plain agar or any other bloodless medium, but there is a point reached after which there is no further loss of virulence no matter how much sub-

transplanting is done. In fact, *H. pertussis* never becomes totally avirulent even though the organism may lose its power to produce massive agglutinins in the rabbit's serum and may change in morphology to 5 to 10 times the size of the originally isolated organism (55 strains studied). From a virulence standpoint, this final stage of lessened virulence could be termed the fixed or R stage of the organism's existence. Since the changed organisms are approximately 5 times the size of freshly isolated ones and one-fifth as virulent there is a possibility that equal numbers of organisms, irrespective of weight, may be equally virulent. Based on virulence tests, *H. pertussis* organisms retain their original characteristics when grown for over 2 years on potato blood agar. There is a modification of this virulence after as few as 10 subtransplants on veal brain or plain agar, but not so soon on chocolate brown agar medium.

M9. A Comparison of the Incidence and Biological Characteristics of the Hemolytic Bacillus coli Recovered from the Intestinal Tract of Healthy Individuals and Patients with Ulcerative Colitis. EDITH E. NICHOLLS, New York Hospital-Cornell University Medical College.

In a previous article the author reported the incidence and biological characteristics of the hemolytic *Bacillus coli* in the stools of healthy individuals (J. Clin. Invest. **13:** 479, 1934). The present report consists of a similar study of samples of feces removed from ulcers by swabs introduced through the proctoscope in cases of ulcerative colitis and a comparison of the results with those found in healthy people.

Fifty smears from 27 patients with ulcerative colitis were cultured for the presence of *Bacillus coli* and the hemolytic type was found in 37 or 74 per cent of the samples. This is only slightly higher than the 64 per cent found for healthy individuals. In the first or single specimens cultured, the incidence of hemolytic *Bacillus coli* was 66 per cent for the colitis cases and 56.2 per cent for the normals. Where two or more specimens from the same individual were studied, the figure rose to 100 per cent for the former and 88 per cent for the latter.

The hemolytic *Bacillus coli* recovered from patients with ulcerative colitis showed no greater virulence for white mice than those recovered from controls.

The *Bacillus coli* from both groups appeared to be heterologous strains having agglutinins more or less in common.

In cases of ulcerative colitis the incidence of hemolytic *Bacillus coli* is

similar to that found in stools of healthy people and the organisms have the same biological characteristics.

M10. The Isolation of Neisseria gonorrhoeae. ALICE D. LEAHY AND CHARLES M. CARPENTER, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The isolation of *Neisseria gonorrhoeae* is attempted in all patients suspected of having gonococcal infections. Observations have been made on the suitability of different media, on various atmospheric requirements and temperature of incubation, and on the value of the "oxydase reaction," described McLeod and his associates (McLeod, J. W., Coates, J. C., Happold, F. C., Priestly, D. P., and Wheatley, B., J. Path. and Bact., **39**: 221, 1934) for identifying gonococcus colonies in mixed cultures.

Chocolate blood agar plates incubated at 37°C. in an atmosphere containing 10 per cent CO_2 yielded the greatest number of positive cultures. Because a few strains failed to grow under these conditions, it was necessary to incubate duplicate cultures at 34°C. in a normal atmosphere.

Prior to the use of this method, 40 cultures of *N. gonorrhoeae* were isolated from 155 patients with gonococcal infections (25+ per cent). By employing all of the above factors, 42 strains were isolated from 74 patients (56+ per cent).

The "oxydase reaction" alone demonstrated 13 of the 42 positive cultures, which could not be identified by direct macroscopic examination of suspected colonies followed by microscopic examination of smears. The use of these procedures resulted in only one failure to isolate the gonococcus from 30 patients when smears from the accompanying inoculum showed the presence of Gram negative diplococci.

M11. Endogenous Infections by Clostridium welchii. LUCILLE C. LYNCH AND PAUL F. CLARK, Department of Pathology and Medical Bacteriology, University of Wisconsin Medical School, Madison

The uncertainty as to the source of the etiologic agent of certain post operative cases of gas gangrene developing at the Wisconsin General Hospital has brought into question the pathogenesis of this type of the disease. The possibility of an endogenous origin of the organism suggests that physical or chemical factors introduced at operation may alter the environment so that the growth of *Clostridium welchii* is stimulated.

Our work has been directed along the following lines:

1. Distribution of *Clostridium welchii* in the animal body.

a. Clostridium welchii was isolated from 12 of 14 human livers obtained at autopsy; 8 of the specimens were removed within 6 hours post mortem. In only one case was liver pathology present.

b. Clostridium welchii was isolated from 8 of 15 livers removed during life from dogs during operative procedure.

c. Clostridium welchii was isolated from the meconium of 10 infants during their second day of life at the Wisconsin General Hospital.

2. Experimental endogenous infections with *Clostridium welchii*.

a. Clostridium welchii was isolated from peritonitis produced in laboratory animals by the intraperitoneal injection of sterile bile.

M12. Effect of Dissociation on Specificity of Hemolytic Streptococci. RUTH TUNNICLIFF, John McCormick Institute for Infectious Diseases, Chicago, Ill.

Earlier papers have shown that hemolytic streptococci isolated during the acute stage and from complicating lesions of scarlet fever and those from erysipelas form distinct immunologic groups, to neither of which belong those from septic sore throat. The streptococci from each of these three diseases were observed to produce distinctive types of colonies on chocolate agar which is not changed in color by typical scarlatinal streptococci, but is turned a vivid green by those peculiar to erysipelas and septic sore throat.

Dissociation of some of these strains produces a variety of colonies including mucoid and rough, which immunologically differ from these in the original culture. Many of these dissociants can be reverted to the original type, colonially and immunologically, by subculturing in dextrose broth.

During the course of scarlet fever not only the specific type of conical colony has been isolated, but also the same nonspecific granular and smooth convex and other types of colonies found in dissociating scarlatinal cultures, with the exception of the extremely rough flat colony. The non-specific strains can in turn be reverted to the type specific for scarlet fever.

Besides opsonic tests used for differentiating typical strains of scarlatinal streptococci from their dissociants, a few toxin neutralization experiments were made on paramecia, with similar results. The toxins from typical scarlatinal streptococci are neutralized by specific scarlet fever antitoxin, while the toxins from the dissociants are not.

During a recent hospital epidemic of scarlet fever 105 cultures of hemolytic streptococci were isolated and studied. Streptococci apparently specific for scarlet fever were identified in cultures from carriers and patients without a rash but suffering from sore throat and infections of the nasal sinuses and the ears. These streptococci were recognized by the fact that they formed conical colonies which did not turn chocolate agar green and were specifically opsonified.

Eight of the 10 strains of opsonically nonspecific hemolytic streptococci from convex and conical colonies which greened chocolate agar, were shown to be dissociants of *S. scarlatinae* when they were reverted to cocci which formed conical non-greening colonies, and were now opsonified specifically by antiscarlatinal streptococcus horse serum.

It is obvious that to determine specificity of bacteria, it is necessary to use both a specific serum and a stable organism and that dissociating strains are unsuitable for either specific immunization or immunity tests. Over-immunization may also produce a nonspecific serum. The opsonic test has the advantage that suspensions for the test can be made from the growth on a chocolate agar plate on which changes in color and type of colony may readily be seen. Fluid cultures for agglutination and toxin-neutralization tests should therefore be subcultured on to chocolate agar just before the experiment to determine whether the culture has dissociated during the incubation period. A dissociating strain will show specific immunologic reactions only if the specific factor predominates.

M13. The Cultural and Serological Characteristics of Minute Hemolytic Streptococci. ELEANOR A. BLISS AND PERRIN H. LONG, The Johns Hopkins University Medical School, Baltimore, Md.

Minute beta hemolytic streptococci have been isolated primarily from the rhinopharynges of normal and diseased human beings. These organisms grow satisfactorily in blood broth and upon 5 per cent blood, neopeptone, beef infusion agar plates. The colonies are very small and the area of hemolysis is of the beta type. The organisms are smaller than the ordinary beta hemolytic streptococci; they tend to occur in short chains and masses. The majority of our strains have *Streptococcus equi*-like fermentation reactions in lactose, mannite and salicin, but certain strains have the pyogenes, infrequens or sub-acidus type of fermentation reactions. The majority of the strains ferment trehalose. None ferments sorbitol. Sodium hippurate is not hydrolyzed and methylene blue is not reduced. A moderate amount of acid is generally

produced in 1 per cent dextrose broth. Preliminary studies of the antigenic structure of these organisms show that, upon the basis of Lancefield's precipitin reaction they fall into three groups. When serological differentiation is attempted by means of agglutination reactions two distinct groups have been outlined. Human beings suffering from purulent infections caused by these minute organisms show agglutinins in their blood serum.

M. 14. The Incidence and Significance of Minute Hemolytic Streptococci. PERRIN H. LONG AND ELEANOR A. BLISS, The Johns Hopkins University Medical School, Baltimore, Md.

Minute beta hemolytic streptococci have been isolated from the throats of 80 per cent of individuals ill with glomerular nephritis and 50 per cent of individuals ill with rheumatic infection. These organisms are rarely found in the throats of individuals ill with chronic disease or acute infections. Their incidence in normal human beings is from one-half to one-third of the incidence of ordinary beta hemolytic streptococci. In both glomerular nephritis and rheumatic infection more throat cultures were positive for minute hemolytic streptococci than for ordinary beta hemolytic streptococci. In many individuals suffering from these diseases, only minute hemolytic streptococci were recovered from throat cultures during the period of investigation. In view of the well known association of these two diseases with beta hemolytic streptococcus infection we feel that these findings assume an added importance. In two instances these minute organisms have been the sole etiological agent in purulent infections in human beings.

M15. Observations on an Epidemic Infection with Hemolytic Streptococcus in an Isolated Group of Rheumatic Subjects. A. F. COBURN, Columbia University, New York.

An outbreak of respiratory disease at The Pelham Home during the spring of 1934 made it possible to study the herd response of 25 rheumatic children to infection with a single pathogenic agent. This organism gave the fermentation reactions of *Streptococcus pyogenes*, according to Holman's classification, and was a strong toxin producer. Familiarity with the patients' previous rheumatic attacks led to the conclusion that this strain was effective in initiating a more intense activity of the rheumatic process than had been previously experienced by these individuals. No evidence was found either for the existence of a refractory state of the host or for protection from rheumatic fever by

good living conditions. There was a close correlation between the development of the rheumatic recrudescence and the stimulation of the immune mechanism as judged by serial antistreptolysin determinations of the blood serum.

M16. Sensitivity to Nascent Phage as a Character for the Differentiation of the Beta Type of Streptococci. ALICE C. EVANS, National Institute of Health, Washington, D. C.

Sensitivity to the 4 types of streptococcus phage described by the writer in Public Health Reports (now in press), offers a new point of view from which the relationships of hemolytic streptococci from various sources may be studied. The usefulness of phagological reactions as a supplement to other characters may be illustrated by the aid they have given in the classification of *Streptococcus equi*.

Failure to ferment lactose is the character by which the streptococcus of strangles is generally differentiated from other hemolytic streptococci, yet some investigators state that certain strains of *Streptococcus equi* ferment lactose. When this disagreement is examined in the light of phagological reactions an explanation is at hand.

Streptococcus equi is distinguished from other streptococci by its sensitivity to all of the 4 types of phage in the nascent state (that is, in the presence of a sensitive strain). In a large collection of strains now being studied, certain strains which fermented lactose must be regarded as belonging to a variety of *Streptococcus equi* on account of their ability to produce strangles in horses and their sensitiveness to the 4 types of phage. Five out of 22 strains from strangles fell into the lactose fermenting variety.

Not one of the collection of over 300 strains of hemolytic streptococci from a great variety of human diseases fully agreed with any of the streptococci from strangles.

M17. A New Method of Staining Bacteria and its Application to a Study of Streptococci and the Viruses of Poliomyelitis and Encephalitis. EDWARD C. ROSENOW, The Mayo Foundation, Rochester, Minn.

The method consists essentially of staining deeply the thoroughly fixed film with a basic dye, such as methyl violet, washing with water, adding in turn, Gram's iodine solution and a 2 per cent aqueous solution of safranine, and washing with water. Counterstaining with safranine after decolorization with alcohol, as in the Gram method, or treatment

either with the iodine solution alone or with the safranine solution alone, does not suffice. The combination of iodine and safranine is necessary to stain the delicate forms as found in filtrates, and the capsules of mature organisms.

By this method unmistakable oval cocci and diplococci, usually singly but occasionally in short chains, have been found consistently in filtrates of emulsions of glycerolated spinal cords of persons and monkeys that died of acute anterior poliomyelitis; in filtrates of the brains of rabbits and mice that succumbed to inoculations of the viruses and streptococci from poliomyelitis, epidemic encephalitis and herpes-encephalitis; in direct smears of spinal fluid of persons and monkeys in the acute stage of poliomyelitis, and in filtrates of cultures of the streptococcus in mediums such as "K" medium, Noguchi's medium and chick-mash medium. Organisms were not demonstrable in control filtrates and spinal fluids. Cultures of filtrates were usually negative.

All species of cultivable bacteria thus far stained by this method have revealed capsular envelopes of varying thickness and density.

M18. The Serological Differentiation of Pathogenic and Non-Pathogenic Strains of Hemolytic Streptococci from Parturient Women. REBECCA C. LANCEFIELD, Hospital of Rockefeller Institute for Medical Research, New York, and RONALD HARE, Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Hospital, London.

The differentiation of hemolytic streptococci which are likely to do harm during childbirth from those which are not is based on previous studies showing that broad groups of hemolytic streptococci can be distinguished serologically by the polysaccharide common to each group.

In Queen Charlotte's Hospital, London, hemolytic streptococci were obtained from 13 of 855 women ante partum, and from 85 of another 837 post partum; only one woman from these two groups had definite puerperal infection. Forty-five additional strains from 45 women with puerperal sepsis were also studied. These strains were classified into groups by the precipitin reaction using extracts, prepared by heating the streptococci with N/20 HCl, and antisera from rabbits immunized with formalinized cultures. All strains from patients with definite puerperal infection (except one associated with *Staphylococcus aureus*) belonged to Group A; whereas only one Group A strain was found among the cultures from afebrile patients, and all other strains from afebrile patients fell into groups other than Group A. It is concluded, therefore, (1)

that hemolytic streptococci may be harbored in the vagina before or after delivery without causing disease provided they belong to serological groups other than Group A, (2) that Group A strains are the only hemolytic streptococci capable of causing definite puerperal infection in the human species, and (3) that infection almost invariably occurs if Group A hemolytic streptococci are present in the vagina.

The serological method of identifying hemolytic streptococci potentially pathogenic for man is also applicable epidemiologically wherever non-pathogenic streptococci of other groups may be encountered simultaneously with Group A strains, as in the separation of human and bovine strains in milk during epidemics of septic sore throat, or in milk borne epidemics of scarlet fever, or in routine examinations of milk to exclude pathogenic hemolytic streptococci.

M19. The Dissemination of Human Pathogenic Streptococci Through the Cow's Udder. D. J. DAVIS, University of Illinois College of Medicine, Chicago.

The numerous milk borne outbreaks of disease have focused attention on the mechanism of infections of the cow's udder. Tubercle bacilli, *Brucella abortus* and possibly paratyphoid bacilli reach the udder from the blood stream or tissues of the cow. Other bacteria, notably streptococci and possibly staphylococci, may reach the udder through the lactiferous ducts of the teats. Outbreaks of septic sore throat traced to milk at times have followed teat injuries. There is no evidence that hemolytic streptococci reach the udder from the blood or tissues. It is assumed, therefore, that streptococci have been implanted on the skin of the teat from some human source and have ascended the duct to the milk cisterns. Experimentally hemolytic streptococci causing outbreaks of sore throat when placed on the teat will cause a definite streptococcus mastitis in a short time.

Sections made of the cow's teat reveal the dense squamous cornified epithelium lining the lactiferous duct around which is the muscle sphincter. The epithelial surface of the duct which varies in length from 0.6 to 1 cm. is rough and irregular furnishing small pockets and crevices in which the milk may be retained. It is along such a channel that the streptococci by growth, possibly aided by manipulation, ascend. The sphincter is adequate in most teats to prevent leakage. Not infrequently, however, it becomes incompetent and as the tension of the milk in the cisterns increases the milk trickles through creating a direct route through the meatus to the exterior where bacteria may enter.

As to whether a negative udder pressure and, therefore, suction during the process of milking may occur was tested in a series of 13 milch cows by inserting the end of the teat in a dye (carbol fuchsin) as the pressure on the teat was released. The cows were then immediately slaughtered and the teats examined. The dye did not ascend in the lactiferous duct in any instance more than one half the distance to the cistern, even in a leaking teat.

In an udder in the site of experimental mastitis no changes of any importance were noted about the meatus or along the duct epithelium to indicate involvement of the lymphatics or subepithelial tissue. The main body of the udder in experimental human streptococcus mastitis reveals extensive inflammatory changes.

The streptococci used in the experiments, isolated from outbreaks of septic sore throat, were encapsulated and revealed large moist watery or mucoid growth. These streptococci, sometimes called *Streptococcus epidemicus*, ferment trehalose but not sorbitol. We assume they are human in origin. According to studies of Dawson, these streptococci may represent mucoid variants.

In most outbreaks of udder borne septic sore throat, no scarlet fever appeared. Erysipelas as a complication occurs in a few cases. In udder borne scarlet fever, as in the outbreak in New Jersey, most cases were typical and relatively few cases of septic sore throat appeared.

These accidental human experiments of udder borne disease on such a vast scale and of such great virulence should be valuable criteria in determining the specificity of streptococci. Practically all biological and laboratory tests except possibly the specific toxin test of scarlet fever streptococci are more or less variable. This is true even of hemolysis, as Pilot has shown.

M20. Laboratory Infections Due to Brucella in the United States. K. F. MEYER, Hooper Foundation, University of California, San Francisco.

Seventeen organizations or institutes, in answer to a questionnaire, reported for the period 1922 to 1934 a total of 57 clinically recognized infections directly traceable to routine or research work with brucella organisms. The number of cases stands entirely in proportion to the extent of the investigative work which has been conducted by the laboratory. Five well known centers of brucella research had over a period of from 7 to 12 years, from 5 to 10 infections. In 45, 6 per cent of the clinical cases, the causative organism was isolated in blood cul-

tures. As might be expected from previous experiences, *Br. melitensis* was the offender in 53.8 per cent, the suis type in 42.3 per cent and the abortus bovis type in 3.9 per cent of the successful cultures. The course of the disease was mild in about one-half of the patients and the duration from 1 to 2 weeks. About 20 per cent passed through the disease in an ambulatory stage. Unfortunately, an equal number had a stormy, prolonged illness. The melitensis infections have been particularly serious, in one instance leading to a brucella cholecystitis. It may be mere coincidence but the infections were much more severe in the women than in the men. Preventive vaccination, as might be expected, afforded no protection.

M21. Brucella Infections in White Mice. CHARLOTTE SINGER AND E. B. SHAW, Hooper Foundation, University of California, San Francisco.

In a comparative series it was found that inoculation with 300 million *Brucella* bacteria of the suis type, human origin, was fatal to white mice within four days. Inflammatory and degenerative changes in the mice surviving 30 million, 3 million and 300,000 bacteria were entirely proportional to the number of organisms administered.

Mice immunized intraperitoneally with vaccine survived a mean inoculation of 400 million organisms of suis type. All controls died. There were no abortions; there was relatively little anatomical change, principally in spleen and lymph nodes. Infection persisted for 14 months.

In a similar series cross protection was tested with small numbers of organisms. The immunized animals re-infected with small inoculum freed their organs slightly better than controls. Judging by the persistence of infection the melitensis is slightly more invasive than suis or bovis types. There is no striking difference in the sterilization of tissues irrespective of the number of organisms inoculated. Positive cultures were obtained for 104 days (longest period tested). Immature mice 3-4 weeks old were more capable of disposing of small and large doses of living organisms of any type, regardless of whether the mothers were infected or immunized.

M22. The Preparation of Diphtheria Toxoid Adsorbed on Calcium Phosphate. AUGUSTUS WADSWORTH, JAMES J. QUIGLEY AND GRETCHEN R. SICKLES, Division of Laboratories and Research, N. Y. State Department of Health, Albany.

When the early work of Roux and Yersin in 1899 and the more recent work of Abt and of Smith was repeated, it was found necessary to develop the procedure in certain particulars in order to insure practical results in the recovery of diphtheria toxoid, and also of the toxin, by precipitation on calcium phosphate. The following method was adopted and has been found to give a yield of 90 per cent or over in flocculating units: To 100 cc. of diphtheria toxoid are added 10 cc. of a 10 per cent solution (1 gram) of calcium chloride, followed by 20 cc. of a 10 per cent solution (2 grams) of disodium phosphate. An excess of calcium chloride is essential to avoid the elution of the toxoid. Complete adsorption requires about 20 minutes at room temperature. The supernatant liquid (pH 6.2±) is removed; the precipitate is washed twice with water, three times with physiological salt solution and suspended in salt solution. The resulting product should be free from calcium chloride with the pH approximately 7.0. The Kjeldahl nitrogen is reduced about 90 per cent. Calcium and phosphorus determinations indicate that the precipitate consists of about 70 per cent tricalcium phosphate and 30 per cent dicalcium phosphate. This preparation remains in suspension for a longer period than does alum-precipitated toxoid. Recovery is always about 90 per cent, while less than 10 per cent of the original nitrogen is present. The final reaction is about neutral; that of the alum preparations is acid. For flocculation tests, the precipitated toxoid is dissolved in diammonium citrate solution (pH 5.0).

A toxoid was prepared by the usual method of Ramon from a toxin produced in a modified infusion-free peptone medium containing maltose and sodium acetate, by a strain of the diphtheria bacillus the toxigenic activities of which had been developed, after isolation in this laboratory, to a degree equivalent to that of the standard strain of the diphtheria bacillus, Park-Williams No. 8. The toxoid was precipitated by the foregoing method with a recovery of 100 per cent of the flocculating units. The antigenic activity of this calcium-precipitated toxoid, as shown by the antitoxic content of the blood serum of guinea pigs after immunization, was equal to that obtained with alum preparations of toxoids produced by this strain and by the standard No. 8.

M23. A Method for the Production of Staphylococcus Toxin and Toxoid.
GEORGE F. LEONARD AND AUGUST HOLM, Biological Laboratories, E. R. Squibb and Sons, New Brunswick, N. J.

Increased interest in the use of a *Staphylococcus* exotoxin and toxoid

in the prevention and treatment of staphylococcal infections has demonstrated the need of a simplified method for its preparation on a production scale.

The culture is grown in a semi-synthetic culture medium, in an atmosphere of carbon dioxide-oxygen, and in an anaerobic drum which has been especially devised for this purpose. Methods are described in detail for the production and titration of the toxin, and for the evaluation of the antigenicity of the toxoid.

M24. Studies on the Standardization of Gas Gangrene Antitoxin (Oedematiens). IDA A. BENGTSON, National Institute of Health, Washington, D. C.

A study of 17 strains of *Cl. oedematiens* indicates rather wide variation in the toxigenic properties. The filtrates of certain strains failed to kill mice in 0.5 cc. amounts while the filtrates of others were fatal in 0.01 cc. amounts. One of the most toxigenic strains was used in the preparation of a large volume of toxin which was to be used as a standard toxin. The medium used consisted of 3 parts of beef infusion broth and one part of chopped beef to which was added 5 per cent of sterile normal horse serum. The reaction of the broth was adjusted to pH 8.4. After the addition of the meat and sterilization the reaction fell to pH 7.0. After 3 days' incubation the growth was filtered and the toxin was precipitated by saturating the filtrate with ammonium sulphate. The yield of toxin was over 700 grams from 60 liters of filtrate.

The minimal lethal dose of the dried toxin for a 20 gram mouse was in the neighborhood of 0.02 mgm. The "test dose" of toxin against 0.02 of one international unit of oedematiens antitoxin as proposed by Dr. Madsen of the Statens Seruminstitut of Copenhagen was found to be 2 mgm. (100 M.L.D.'s of the toxin). Tests were carried out on mice (intramuscular inoculation) and on guinea pigs (intracutaneous inoculation).

M25. Agglutination with B. proteus X19 in Specimens Submitted for Other Serological Tests. RUTH GILBERT AND MARION B. COLEMAN, Division of Laboratories and Research, New York State Department of Health, Albany.

Since typhus fever and Rocky Mountain spotted fever have been recognized with increasing frequency in this country, and relatively few cases have been reported as occurring in New York State, an investigation was undertaken to determine the incidence of agglutination with

B. proteus X19 in blood submitted to be examined for evidence of typhoid or undulant fever. Reactions were obtained with *B. proteus* X19 with about 10 per cent of approximately 5,000 specimens examined. The data available indicate that most of the patients whose sera agglutinated this microörganism were not believed by the attending physician to have typhus or Rocky Mountain spotted fever at the time the specimens were collected. The results of the study suggest the need of a thorough investigation when reactions with *B. proteus* X19 occur.

M26. Oral Heterophile Immunization. GEORGE E. ROCKWELL AND HERMAN C. VAN KIRK, Department of Bacteriology, University of Cincinnati.

The object of these experiments is to show that oral administration of certain Forssman's antigens will stimulate the development of a heterophile immunity to such an extent that it offers a formidable protection against certain virulent organisms. The experiments also indicate the duration of this protection.

Rabbits were given orally one billion heat killed, rough variant type I pneumococci (D.R.-I) in 5 cc. of water one hour before the rabbits were fed in the morning. Their heterophile immune response and the duration of this response was shown first by frequent titration of their serum for sheep cell hemolysin; secondly by intradermal inoculation with several lethal doses of rabbit virulent type II pneumococcus.

Five days after the last of 8 oral treatments the rabbits showed a heterophile titration from 240 to 480 units per cubic centimeter of serum. But after a rest period of 9 to 12 days this titration would drop to 30 or 60 units. If after a 12 day rest period another oral treatment was given within 12 hours, the heterophile titration would increase from 30 to 120 units or better.

When the above rabbits were inoculated intradermally with several lethal doses of rabbit virulent type II pneumococci the following results were obtained:

(1) Those that received this inoculation within 5 days of the last oral treatment *lived.*

(2) Those that received this inoculation 9 days or more after the last oral treatment *died.*

(3) Those that had a rest period of 12 days after the last oral treatment before inoculation, if given another oral treatment with the inoculation, had about 25 per cent mortality.

M27. The Wassermann Reaction and the Kahn Test in Leprosy. M. H. SOULE, Hygienic Laboratory, University of Michigan.

The specificity of the various laboratory tests for syphilis with the sera of individuals infected with Hansen's bacillus has frequently been questioned. There is considerable evidence that leprosy interferes with these methods. By way of explanation it has been stated that a positive reaction in leprosy lies in the simultaneous occurrence of syphilis or yaws and in mistakes in clinical diagnosis. As long as the amelioration of leprosy appeared to be hopeless there was little need for more than passing scientific interest into the part that the disease might play in non-specific serological reactions. With the accumulation of evidence that chaulmoogra oil and its derivatives have unquestioned therapeutic value it becomes increasingly important for the serologist to be able to furnish the clinician with data which will aid in the wise interpretation of the tests for syphilis in the presence of leprosy. With this end in view the sera from 669 cases of leprosy were tested by means of the Kolmer Wassermann complement fixation reaction and the Kahn precipitation test. These two procedures were selected because of a familiarity with them and the fact that experience has shown the advisability of using a Wassermann test along with a precipitation test of proven sensitiveness and specificity. In every instance a special effort was made by the clinicians to establish the absence of syphilis or yaws. The data are presented in the following table:

The Kolmer-Wassermann and Kahn reactions with the sera of lepers (Filipinos), presenting no evidence of syphilis or yaws

TEST	NUMBER OF SERA TESTED	STRONGLY POSITIVE	POSITIVE	DOUBTFUL	NEGATIVE	PER CENT NEGATIVE	PER CENT POSITIVE
Kolmer	615*	109	5	14	487	81.5	18.5
Kahn	615	121	70		424	69.0	31.0
Sera in which the two reactions were identical		100	5		407		

* 526 males and 89 females.

It is concluded that leprosy *per se* is responsible for the positive reactions.

M28. Native Versus Denatured Bacterial Antigens. H. M. POWELL AND W. A. JAMIESON, The Lilly Research Laboratories, Indianapolis.

Following experimental devitalization of bacteria with the non-coagulating antiseptic, Merthiolate, as a method for preparation of more nearly natural antigens, we have continued by the methods of Krueger, accomplishing both devitalization and fragmentation of bacteria by mechanical means comprising grinding and ultrafiltration. Such ultrafiltrates preserved with Merthiolate consist almost entirely of undenatured bacterial antigen ("U B A").

Utilizing carefully selected cultures of *H. pertussis* and other bacteria corresponding to those from which ordinary vaccines are made, we have prepared U B A in increasingly large quantities for clinical evaluation in pertussis, paranasal sinusitis, gonorrhea, etc. The earliest clinical reports on such U B A have appeared or are in press.

Laboratory standardization of different antigens has been on the basis of nitrogen value, and pertussis U B A of 10 mgm. nitrogen per 100 cc., for example, have appeared to be of an optimum concentration for human use. Precautions against denaturation as suggested by Dr. Krueger comprise the use of alkaline buffered bacterial suspending medium, grinding at slow speed and ultrafiltering at a rapid rate, both at cool temperatures, and finally control of all preparations for unsuspected denatured protein by isoelectric precipitation.

As compared to ordinary vaccine, U B A is quite free of bacillary metabolic products and culture media ingredients. It is quite atoxic and optimum human doses represent much greater numbers of original bacteria than do regular doses of bacillary vaccine. The immunological response to U B A should be specific to native bacillary substance, and seemingly cannot be evaluated on the basis of conventional serum antibody production.

M29. Simultaneous Immunization with a Mixture of Ten Kinds of Laked Blood. EDNA DELVES, John McCormick Institute for Infectious Diseases, Chicago.

Precipitin, agglutinin and hemolysin production following the simultaneous immunization of 8 rabbits with an antigenic mixture of 10 kinds of laked blood has shown that rabbits may respond to such multiple immunization by producing precipitins for a large number of different blood proteins and hemoglobins at the same time that they produce agglutinins and hemolysins for red blood cells. By absorption experiments the agglutinin and lysin for a particular kind of red blood corpuscle could be removed with little or no reduction in the agglutinins for other corpuscles or in the precipitins for homologous and heterol-

ogous antigens; and a particular hemoglobin precipitin could be removed by specific absorption with little or no reduction in the precipitins for other hemoglobins or in the agglutinins and lysins for homologous and heterologous corpuscles.

M30. The Fate of Specific Antigen in the Immunized Animal: Extent of Its (1) Temporary Retention in Area of Injection, (2) Diffusion from this Area, (3) Destruction in this Area, and (4) Destruction following Diffusion. R. L. KAHN, E. B. MCDERMOTT AND S. D. SATTLER, University of Michigan, Ann Arbor.

When specific antigen is injected into a tissue of an immunized animal, some fixation takes place locally between the tissue and the antigen, probably by colloid chemical union of serum and antigen. The capacity of the tissue to combine with antigen determines the extent of local retention of the antigen and is dependent upon at least three conditions: (1) the state of immunity of the animal, (2) the tissue injected, and (3) the dose of antigen employed.

The antitoxin-toxin method of measuring the reacting capacities of different tissues of an immunized animal to specific antigen was employed in the present investigation. Animals are first immunized with horse serum and the capacities of the tissues to combine with or retain injected antigen are determined by the use of standardized horse serum diphtheria antitoxin in place of horse serum, the antitoxin being regarded as specific antigen.

(1) If into the skin of a rabbit immunized by two doses of horse serum, 1000 units of horse serum antitoxin and 50 M.L.D. of diphtheria toxin, are injected simultaneously about 5 cm. apart, the animal will succumb to the toxin. If instead, the antitoxin is injected into the skin 24 hours before the toxin, 750 units are sufficient to save the animal from toxin death. The fact that 750 units of antitoxin, when injected 24 hours before the toxin, are sufficient to save the rabbit from toxin death, would indicate that when the 1000 units were injected simultaneously with the toxin, approximately 250 units were temporarily held back in the injected area. This temporarily retained antitoxin apparently did not reach the rapidly diffusing toxin fast enough. It appears that temporary retention of part of the antigen takes place, when the total quantity injected somewhat exceeds the antigen-combining capacity of the tissue.

(2) When the excess of antitoxin, such as 1500 units, is injected, simultaneously with 50 M.L.D. of toxin, into the skin of a horse serum-

immunized rabbit, antitoxin diffuses from the area of injection with sufficient rapidity to neutralize the toxin *in vivo*. In another experiment, 5 rabbits similarly immunized with horse serum are injected subcutaneously with 0.01, 0.1, 1.0, 5.0 and 10.0 cc., respectively, of this serum. The rabbits receiving from 0.01 to 1.0 cc. doses show inflammatory responses of increasing intensity and no horse serum in the blood stream. The rabbit receiving 5.0 cc. will show only a mild local response and the one receiving 10.0 cc., no local response. Horse serum is present in the blood stream in both of the latter. The diffusion of antigen from a tissue of an immunized animal can not be said to result solely from the injection of an excessive dose. When a small dose is injected, some slight diffusion must also take place, even though not detected thus far. This view is based on the assumption that the increase in immunity of an animal receiving repeated injections of antigen into some tissue, is due to traces of antigen that reach the circulation.

(3) The destruction of antigen within the injected area of a tissue of an immunized animal was demonstrated by two methods: (*a*) A method which showed the gradual disappearance of antigen from the area of injection; and (*b*) the antitoxin-toxin method above. By the latter method it was shown that unless the amount of antitoxin injected exceeds the specific combining capacity of the tissue, the antitoxin does not diffuse appreciably from the injected area in horse serum-immunized rabbits. This would indicate local destruction of the antigen, presumably by proteolysis.

(4) Protein antigen, injected intravenously with consequent ready diffusion rapidly disappears, most likely by proteolysis. Fifty units of antitoxin injected intravenously are insufficient to protect a horse serum-immunized rabbit from 50 M.L.D. of toxin, injected simultaneously into the skin, while 5 units of antitoxin are sufficient to protect a normal rabbit under the same conditions. Seventy-five units would have saved the animal, suggesting that the 50 units must have undergone such a change as to render them incapable of neutralizing the toxin. If the antitoxin is given 7 days before the toxin, as many as 7500 units are insufficient to protect a horse serum-immunized rabbit from 50 M.L.D. of toxin. These findings indicate that, as in the case of localized antigen, diffused antigen must also be destroyed with considerable rapidity in specifically immunized animals.

M31. Lessons in Laboratory Diagnosis from the Recent Outbreak of Amebiasis. FRED O. TONNEY, Board of Health, Chicago, Ill.

The common amoebae of the human intestinal tract, viz.: *E. histolytica, E. coli, E. nana, Iodamoebae butchlii* and *Diendamoeba fragilis*, are treated from the standpoint of the principal diagnostic points for quick identification, in the order of their importance. The trophozoites of each form are considered under the heading of motility, the nuclei, cell inclusions, the character of protoplasm and size. The cysts of each amoeba are taken up in the same manner, under the headings, chromatoid bodies, the nuclei, size and general considerations.

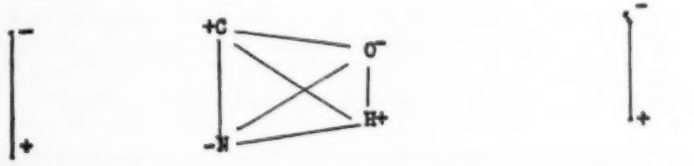
Other parasitic forms and cellular bodies found in feces are: *Trichamonas intestinalis, Guardia hominis, Chylomastix mesnili, Blastocystis hominis*, rounded epithelial cells, large macrophages, mucous cells, fat globules and vegetable cells.

There follows a laboratory guide, in outline form, of the essential differential points needed for the practical diagnosis, based on the author's recent experiences.

M32. Calculated Energy Effects as Suggestive of a Mechanism of the Influence of Antigen on Antibody Formation. ALLEN E. STEARN, University of Missouri, Columbia.

The work of Landsteiner and others has shown the importance of specific chemical groups on an antigenic molecule in determining the nature of the antibody formed, and thus, ostensibly, in the mechanism of antibody formation. Unless the antibody is itself a hydrolytic product, or modified hydrolytic product, of the antigen (in which case it is practically impossible to visualize any basis for antigen-antibody reaction from considerations of energetics), then the latter must function in some enzymatic capacity in the formation of antibodies. Energy calculations show that this is a real possibility. If we grant the claim of Northrop that at least certain enzymes are protein in nature, then some of the chemical groups present in proteins must be able to exhibit prosthetic activity. The function of any enzyme is to lower the activation energy of a reaction. If the enzyme be a hydrolytic one, then the lowering of the activation energy for hydrolytic decomposition and that for re-synthetic "dehydration" must be precisely the same, which of course means a catalyzing effect on the formation of polypeptids, etc. In this paper an investigation is made, from energy considerations, to see whether any of the chemical groups commonly associated with proteins (or groups of similar nature) might lower the activation energy of the hydrolytic breaking of a carbon-nitrogen bond, the one involved in

the peptid linkage, and if so what the mechanism of the effect might be, bearing in mind that these groups themselves should not be altered. The chemical groups specifically studied were ≡C—O—, ≡N=O, and =N—H. These are all electric dipoles, and it is here shown that by a proper placing of these (or similar) dipoles near a reacting configuration of atoms, the reaction velocity, depending on dipole strength, orientation, etc., may be either decreased or increased by amounts as much as several thousand times the "uncatalyzed" velocity in the cases investigated. The effect described is, of course, not limited to the groups studied but is a general one. Diagrammatically it can be pictured as follows:



Dipole. Position a. **Configuration of reacting atoms.** **Dipole. Position b.**

So long as the dipole does not approach close enough to become part of the reacting system its effect is a purely electrostatic one, and this may be either to decrease or increase the potential energy of the four atom reacting system. As diagrammed above, the dipole in position *a* would increase the potential energy of the reacting system by tending to pull the C—N away from the O—H, and thus it would act as a *negative* catalyst. Were this dipole in position *a* turned end for end the effect would be reversed. Thus the C—N would be repelled and the O—H attracted by it, causing them to come more easily together, i.e., facilitating their approach and catalyzing their reaction positively. As drawn in position *b* the dipole will be seen to catalyze positively the reaction. Quantitatively the quantum mechanical approach as outlined by the author last year was employed. The present paper does not treat specifically the mechanism of the placing of these dipoles. Ostensibly this will come from a chemotactic or haptophoric bonding between other groups of the enzyme molecule and other groups of at least one of the reacting molecules. It is planned to treat later this problem specifically.

M33. The Spectrographic Identity of Horse Euglobulin and Pneumococcus Antibody Solution Purified According to Felton. CALVIN B. COULTER, ELVIN A. KABAT AND FLORENCE M. STONE, College of Physicians and Surgeons, Columbia University, New York.

Using a hydrogen discharge tube as a source of continuous illumination in the U. V., and a quartz spectrograph, it has been found possible to resolve the ultraviolet absorption spectra of various proteins into 10 distinct bands, whose maxima have the same wave lengths in all the proteins examined. These were crystalline egg and serum albumen, thyroglobulin, horse euglobulin and pseudoglobulin. The intensities of the individual bands, however, vary in the different proteins so that it is possible to characterize a given protein by its spectral absorption curve. The absorption curves given by (*a*) normal horse euglobulin and (*b*) pneumococcus antibody solutions prepared according to the method of Felton (Felton, L. D., Jour. Immun., **21**: 341, 1931) and containing 65–70 per cent of specifically precipitable nitrogen are identical.

M34. Some Oxidation and Reduction Reactions of Pneumococcal Hemolysin. BARNETT COHEN AND HARRY SHWACHMAN, The Johns Hopkins Medical School, Baltimore.

1. With a wider variety of reagents, we have confirmed the observation of Neill (J. Exptl. Med., **39**: 745, 1924) that pneumococcal hemolysin activity may be inhibited by oxidation and restored by reduction. Oxidizing agents such as air, H_2O_2, iodine, ferricyanide, benzoquinone, SeO_2, azochloramid all cause more or less reversible inactivation. Reducing agents such as $Na_2S_2O_4$, H_2S, cysteine, reduced glutathione, thioglycolate, cyanide and ascorbic acid reactivate the oxidized hemolysin.

2. The following evidence leads to the inference that a thiol grouping may be essential to the lytic activity. Cu_2O, which reacts rapidly with SH-compounds to form mercaptides, rapidly and reversibly inactivates the hemolysin. C_6H_5HgOH and $ClCH_2HgCl$, which also can form mercaptides, also inactivate the hemolysin reversibly. $(CH_2I)_2Hg$, which cannot form a mercaptide, does not inactivate. The rates of inactivation by certain of the oxidants are suggestive of their respective actions on thiol compounds.

3. These observations which were recently reported (J. Biol. Chem., **107**: 257, 1934) are in remarkable agreement with the findings by Hellerman and Perkins (J. Biol. Chem., **107**: 241, 1934) on the behavior of the hydrolytic enzymes, urease and papain.

4. The foregoing experiments were directed toward the elucidation of the specific chemistry of the lysin. In addition, the results of some preliminary tests on the suspected active thiol grouping made with the oxidants of reversible oxidation-reduction systems are of interest. It was found that inactivation occurs with 2,6-dichlorophenol indophenol ($E'_0 = +0.19$, pH 7.4) at a rate comparable with that produced by ferricyanide ($E'_0 = +0.43$). With 1,4-naphthoquinone-2-sulfonate ($E'_0 = -0.13$) the inactivation is very much slower, while with phthiocol ($E'_0 = -0.21$) it was not detectable. These observations are offered with all reserve, but they are highly suggestive.

M35. The Fraction of the Pneumococcus Antigenic for Human Beings.
LLOYD D. FELTON, Harvard Medical School, Boston, Mass.

The work is an extension of that reported last year in an attempt to isolate the immunizing substance or substances of the pneumococcus antigenic to man. Various water-soluble fractions of the pneumococcus were tested in human beings by a single subcutaneous injection of 2 mgm. dissolved in physiological salt solution. All preparations were sterilized by passing through a Mandler filter. Reactions following injection of this group of samples may be considered mild. However, with one sample 0.7 per cent of a group of 3200 individuals developed what might be called a severe reaction. None in the group however were so incapacitated as to interfere with their regular activity. Immunity was tested for by the estimation of the amount of protective antibody in the serum 14 days to 3 months after injection, and in a few cases after 10 months. In a small group of over 200 persons, protective antibody was found in all individuals 14 days after injection, in most individuals after 3 months, and in the few tested after 10 months. The amount of antibody found as compared to that already present varied on the average from a 17-fold to a 98-fold increase. All fractions studied were high in polysaccharide content as indicated by the amount of hydrolyzable sugar present and the precipitin reaction. It has not been determined whether the antigenicity is due to this polysaccharide alone, or to a polysaccharide combined with some other chemical, or due to some other cell constituent or product.

M36. Studies on the Specific Characteristics of Syphilitic Blood Proteins.
SETH T. WALTON, City Health Service, Charlotte, N. C.

A series of experiments has been carried out with the purpose of showing the relationship of the condition of the protein equilibrium in

blood serum to the progress of diseased conditions, taking syphilis as a type of disease which has long been classified into a number of more or less well-defined stages clinically.

Studies on some of the physical properties of syphilitic blood proteins has shown that there is a change in the character of the protein molecules in immunity as shown by a different behavior. The surface tension, solubility, refractivity, ionization effects, and cataphoretic behavior of antibody proteins all point to a marked change in the physical state of immune molecules. Since each microörganism presents a different type of surface, with a different chemical and electrical pattern, a correspondingly different antibody is called forth for each.

It may well be assumed that the process of building up an immunity in a body against certain invading microörganisms is equivalent to a rearrangement of the atomic groups in the serum protein molecules in order to produce new molecules which have the power of arresting the enemy. The end products and the extent of this change are dependent upon the character and quantity of the invading agent.

M37. Purification and Concentration of Diphtheria Toxin. MONROE D. EATON AND S. BAYNE-JONES, Yale University Medical School, New Haven, Conn.

In the course of a study of bacterial toxins and a search for chemical antidotes, diphtheria toxin has been purified and concentrated by the following methods.

I. Fractionation with ammonium sulphate at one-third and three-fourths saturation. The fraction obtained with three-fourths saturation contained most of the toxin. Material from this fraction was absorbed on $Al(OH)_3$, eluted with 2.4 per cent disodium phosphate at pH 8.6–9.0, and again fractionated with ammonium sulphate. In successive states this fraction was precipitated with phosphotungstic acid and cadmium chloride. The final solution was dialyzed, and concentrated in vacuo at 20–30°C. Analyses and tests of the solutions were as follows: mgm. N per L_F unit of crude toxin, 0.150; mgm. N per L_F unit of purified toxin, 0.0005; mgm. N per M.L.D. of fresh crude toxin, 0.003; mgm. N per M.L.D. of crude toxin as old as the purified product was 0.006; mgm. N per M.L.D. purified toxin, 0.00005 to 0.0001. The flocculating time of the original toxin was 40 minutes at 45°C. The purified toxin, diluted to contain 72 L_F units per cubic centimeter flocculated in 2 minutes at 25°C. The purified toxin contained approximately 13 per cent N. It coagulated when heated in an acid solution,

gave a positive biuret test and dried down to a yellow glassy residue. The lead acetate test for sulphur was negative and the Molisch reaction was negative or weakly positive. It contained traces of heavy metals. The usual qualitative tests for copper were negative.

II. Precipitation with acetic acid at pH 3.8 according to Watson and Wallace and Watson and Langstaff. Fractionation with ammonium sulphate separated this material into one part which contained some of the toxin but flocculated slowly with diphtheria antitoxin and another part with more of the toxin which flocculated rapidly.

Final yields by these methods have been about 10 per cent of the original amount of flocculating material (toxin and toxoid in the crude broth filtrate). About half of this flocculating material was changed to toxoid during purification. The other half appeared to be unchanged toxin. Solutions remained stable in toxicity during 8 months. The flocculation reaction has been a very convenient, rapid and accurate method for indicating the toxic material in solutions obtained at all stages of these processes of purification.

M38. Two Antigens of High Molecular Weight: Hemocyanins of Limulus polyphemus and Busycon (Fulgur) caniculatum. SANFORD B. HOOKER AND WILLIAM C. BOYD, Evans Memorial, Boston.

The authors have proposed a theory connecting the molecular weight of the antigen with the proportion by weight of antibody to antigen in precipitates made at the equivalence point. In attempting to obtain evidence for or against this hypothesis, it seemed desirable to investigate antigens at the extremes of the molecular weight range. The respiratory pigments of two species, *Limulus polyphemus*, and *Busycon caniculatum*, provide examples of proteins of extremely high molecular weights, 2 million and 5 million, respectively. The latter is the highest molecular weight ever reported. That these values, obtained by Svedberg's ultracentrifugal method, are of the right order of magnitude is shown by the recent measurements of the Adairs and Roches, using the osmotic pressure technic.

The hemocyanins were prepared by repeated precipitation at the isoelectric point. Sera from rabbits injected with them exhibited high titers, gave very rapid flocculation and contained, as determined by the Dean and Webb method of optimal proportions and by chemical analysis, several milligrams of antibody per milliliter; one serum contained as high as 8 mgm. per milliliter. Precipitates made at the equivalence point (also a few at other points) were prepared, washed

and analysed for copper and nitrogen. From the copper values the amount of antigen in the precipitate could be calculated and this checked in a general way with that estimated by immunological test of the supernatant and washings; from this and the total N the ratio of antibody to antigen was computed. The values at the optimum were found to be for *Limulus*, 1.55 (±0.045), and for *Busycon*, 0.68 (±0.049). These agree fairly well with the theoretical values calculated by the authors from their formula, e.g., for *Limulus*, 1.47, for *Busycon*, 0.97. The value for *Busycon* apparently differs from the theoretical by an amount which is statistically significant.

M39. The Biological Activity of a Few Chemical Constituents of Brucella Cells. I. Forest Huddleson and Alfred D. Hershey, Department of Bacteriology and Hygiene, Michigan State College.

Activity of cells before chemical treatment: Brucella cells in the wet or dry state, when injected into normal guinea pigs in amounts as small as 0.02 gram, will produce a rapidly fatal toxemia.

Activity of autolysate from cells: When the cells are permitted to undergo autolysis from their own enzymes in the presence of toluol at 37°C., a colloidal constituent is recovered in solution which, after removal of the cell residue by centrifuging, has been found highly toxic for guinea pigs. It produces a rapidly fatal toxemia similar to that produced by fresh or dried cells. The toxic substance is non-dialysable. The chemical separation of the toxic substance has been undertaken. It has the properties of an albuminoid.

Nucleoprotein fraction: This fraction gives rapid specific precipitation with precipitating sera. There is no specificity in the precipitability of fractions prepared from each of the three species with sera produced by each of the three species.

The nucleoprotein fraction when injected intradermally in a suitable dilution will elicit a local, or local and systemic reaction in brucella sensitive human beings. It has not been found satisfactory for detecting brucella allergy in the guinea pig, rabbit, monkey or cow.

The nucleoprotein fraction prepared from a reversible agglutinating strain of *Br. abortus* (agglutinating with normal sera but not with specific sera) is not precipitable in any dilution with precipitating sera. It is capable of eliciting a specific allergic reaction in brucella sensitive human beings. It is optically inactive.

S substance: The S substance, a non-protein, non-polysaccharide

substance prepared from *Br. melitensis*, is precipitable in a dilution of over 1:400,000. It is non-toxic for experimental animals. Repeated injections of 1.0 cc. of one per cent solution into guinea pigs, stimulates the formation of precipitins which precipitate the solution up to 1:1600 dilution, but no agglutinins for the cells can be detected. Immune opsonins are produced in the guinea pig.

Studies conducted on the Favilli precipitating polysaccharide obtained for *Br. abortus* show that if the chemical separation is carried far enough, the precipitating substance may be obtained free from polysaccharide. The precipitating substance is of the same nature as the S substance from *Br. melitensis*.

Polysaccharide fractions: The polysaccharide fraction recovered from the three species of *Brucella* is inactive biologically.

M40. Application of the H—O Technique of Agglutination to Certain Clostridia. ELIZABETH MCCOY AND L. S. MCCLUNG, University of Wisconsin, Madison.

The H—O technique for serological agglutination of bacteria allows some separation of the factors concerned and therefore reveals new meaning of the agglutination reaction. This is excellently illustrated by an analysis of the difference, yet interrelations, of three species of motile anaerobes, *Cl. acetobutylicum*, *Cl. felsineum*, and *Cl. roseum*. Cross agglutination with differential absorptions shows the following: (1) H factors, separate and species specific, (2) an O factor common to the three species, and accounting for the entire group reaction in two cases, and (3) a complex group reaction involving both H and O fractions between *Cl. acetobutylicum* and *Cl. roseum*. The latter is an interesting case in that the H factor is not a common group H but represents possession by one organism of some of the specific H of the other species.

It was also shown that within one species strains may vary quantitatively with respect to the species specific H factor. That the variant strains were deficient but not devoid of their specific H was shown by agglutinogenic action, by absorptive capacity for H-antibody, and by positive agglutination in a pure H serum of a strain not similarly deficient. The presence of strains of this type in three of the anaerobic species studied so far, *Cl. acetobutylicum*, *Cl. thermosaccharolyticum*, and *Cl. bifermentans*, indicates that this may be a common phenomenon.

M41. Heat Labile and Heat Stable Antigens in the Production of Agglutinins for Various Spore-bearing Anaerobes. L. S. MCCLUNG AND ELIZABETH MCCOY, University of Wisconsin, Madison.

A technique for the production of antisera against the stabilotropic and labilotropic antigens of spore-bearing anaerobes has been developed. Heat-treated cells were chosen for the somatic antigen and the flagellar antigen was the supernatant obtained by long centrifugation, after mechanical agitation, of a suspension of young cells grown in glucose-tryptone broth. Sera prepared against untreated cells were included for comparison. Rabbits were the experimental animals and usually 5 intravenous injections were given on alternate days. The animals were bled on the fourth to sixth day after the last injection and the serum was stored at low temperature. The appropriate antigens for the various tests were prepared in large volumes in normal saline and also refrigerated. No chemical preservatives were used in either antigen or serum. Test readings were made after overnight refrigeration following a four hour incubation period at 52–54°C. The results obtained were entirely comparable in type of agglutination to the descriptions in similar studies of the *Salmonella* and other groups by the English and German investigators.

Various motile anaerobes have been studied with the technique developed. Perhaps the most interesting are the non-pathogenic species, *Cl. acetobutylicum* and *Cl. thermosaccharolyticum*. The thermophilic species, cultivated at 60°C., possessed a heat labile antigen apparently analogous to that of the mesophilic species. *Cl. welchii* was chosen as the non-motile species to be investigated. Somatic sera were prepared from massive doses of heat-treated cells or cells washed free of toxin.

M42. Purification of Suspensions of the Virus of Vaccinia. C. A. Behrens and F. A. Nielsen, Purdue University, Lafayette, Ind.

An efficacious procedure of purifying dermo- and neuro-virus emulsions has been developed.

This method is based upon the separation of the virus from the extraneous material by precipitating the tissue at its iso-electric point.

Acetic, carbonic, citric, lactic, succinic and tartaric acids were found to have very little harmful effect on the virus when used in the small amounts necessary to reach the iso-electric point of the tissue.

Water clear suspensions of the virus have been prepared which have high titers and from which 75 to 85 per cent of the protein has been removed.

M43. An Immunological Study in Laboratory Animals of Thirteen Strains of Equine Encephalomyelitic Virus. BEATRICE F. HOWITT, Hooper Foundation, San Francisco, Calif.

A comparative immunological study was made of 13 different strains of equine encephalomyelitic virus (Meyer, Haring and Howitt). The 11 strains isolated from different parts of the United States could be divided serologically into two groups, an eastern and a western. Of the two foreign viruses, the one from Argentina was classified with the western series while that from Russia was immunologically distinct from the other two groups and varied in several other characteristics.

There was no *in vitro* cross neutralization nor *in vivo* protection between serums of any group when tested against the heterologous viruses.

Animals immunized to each member of the 3 divisions showed a constant tissue immunity within their own groups when tested intracerebrally but cross injection experiments were not conclusive, except for the Russian strain. A certain percentage of guinea pigs immune to the American western strains showed immunity when tested with the eastern and vice versa. There was no cross immunity between the Russian strain and either of the two American groups.

The American eastern strains of virus were more invasive and potent than the western, both by intracerebral and by intradermal inoculation of guinea pigs. The Russian virus was invasive for rabbits intracerebrally but not intravenously.

Comparison of potency was best demonstrated by titration methods.

A discussion is given of the possibility that the strains of virus isolated from horses in the different localities have sprung from a common root stock and should therefore be considered as varieties or subvarieties of the identical virus causing the same clinical disease.

M44. Poliomyelitis. In Vitro Neutralization Tests Using Normal Adult and Convalescent Human Serums. BEATRICE F. HOWITT, Hooper Foundation, San Francisco, Calif.

During the 1934 outbreak of poliomyelitis in northern California, serum was collected both from recovered cases of the disease and from normal adult individuals. The convalescent serums were given therapeutically and the normal pools prophylactically. *In vitro* neutralization tests were made on the pooled lots and on the individual serums with the following results.

Sixteen (66.6 per cent) of 24 undiluted normal serums from profes-

sional donors neutralized a standard amount of poliomyelitic virus while 8 (33.3 per cent) lacked potency.

Pooled undiluted serum from members of the medical staff as well as from normal adult volunteers, all neutralized the same amount of virus. Seven of them neutralized in a 1:10 dilution, 4 out of 6 in a 1:40 while none was positive after diluting 1:80. Sixteen pools of convalescent serum were tested and all but one neutralized when undiluted. Titrations of the latter showed a slightly lower degree of potency than did the normal serums.

Selected lots of serums from house staff members over and under 35 years of age, respectively, as well as from normal adult volunteers of the same age groups all neutralized the virus in a 1:40 dilution of serum.

Titrations of convalescent serums pooled in lots from cases having had poliomyelitis in 1934 and 2, 4, 10, 20 and over 20 years previously showed a positive neutralization in a 1:20 dilution for all the pools except the 20 year group which failed to neutralize in a 1:10 dilution. The pool from the oldest age group, however, was potent when diluted 1:10. Several individual serums from recently recovered cases showed less potency than those from convalescents of longer duration, even over 40 years.

M45. Immunization of Monkeys by Means of Poliomyelitis Virus Adsorbed to Alumina-gel. F. B. Gordon, James A. Harrison and N. Paul Hudson, Department of Hygiene and Bacteriology, University of Chicago.

Rhoads has reported the successful immunization of monkeys by means of virus adsorbed to alumina-gel. Observations in other fields indicate that the antigenicity of a substance may be enhanced by combining it with a colloidal carrier.

We subjected 6 normal monkeys to a series of subcutaneous injections of poliomyelitis virus adsorbed to alumina-gel (Willstätter, Type C). One animal died of poliomyelitis during the course of immunization. The undiluted serum of each of the remaining 5 neutralized the virus *in vitro*. One of the animals was killed accidentally, leaving 4 which were given intranasal inoculations of virus; 3 developed poliomyelitis and the fourth later succumbed to an intracerebral virus inoculation.

A comparable series of 6 normal monkeys was immunized by subcutaneous injections of the eluate of virus-alumina-gel complex. All survived the immunizing injections and the serum of each neutralized the virus. All were given an intranasal inoculation of virus and 5 developed

poliomyelitis. The sixth was also refractory to a subsequent intracerebral virus inoculation.

For control purposes 2 normal animals were injected subcutaneously with alumina-gel which had been shaken with normal monkey nervous tissue, the technic being the same as that used for preparing the virus-alumina-gel complex. Both serums failed to neutralize the virus. After intranasal inoculation only one animal was attacked but the second succumbed to a later intracerebral virus inoculation.

M46. Purification of the Virus Neutralizing Fraction of the Serum of Poliomyelitis Convalescents. JAMES A. HARRISON, Department of Hygiene and Bacteriology, University of Chicago.

A number of workers have shown that bacterial antibodies may be adsorbed to such inert materials as kaolin, chalk, ferric hydroxide, etc. We have attempted to adsorb to an alumina-gel the virus neutralizing substance of convalescent human poliomyelitis serum.

The supernatant of slightly acid (pH 6.5) mixtures of aluminum hydroxide (Willstätter, Type C) and convalescent human serum contained an insufficient amount of antibody to neutralize a test dose of virus in 10 of 13 trials. The alkaline (pH 7.4) washings of the gel adsorbates neutralized test doses of virus in 9 of 11 trials. In the only quantitative experiment tried, the eluate was neutralizing in approximately as high a dilution as was the original serum sample. Similar eluates of normal monkey serum did not neutralize the virus. Biuret tests run on serial dilutions of gel-absorbed serum and eluted antibody indicated a considerable diminution of protein in both fractions.

M47. Failure to Infect Monkeys with Poliomyelitis Virus through Isolated Intestinal Loops. EDWIN H. LENNETTE AND N. PAUL HUDSON, Department of Hygiene and Bacteriology, University of Chicago.

Four *rhesus* monkeys, with Thiry or Thiry-Vella fistulae at various levels of the intestinal tract, were given virus instillations into the isolated bowel loops. Two of these animals received a total of 40 cc. of 20 per cent crude virus-cord emulsion over a period of one week; all received, over a 4-month period, from 8 to 10 grams of *whole* glycerolated or fresh virus-cord. After a brief rest period, 3 consecutive daily instillations of 10 per cent virus-cord emulsions were given, each instillation being preceded by a preliminary flushing out of the isolated bowel with phosphate buffer solution, pH 5.0. No poliomyelitis occurred at any stage of the prolonged treatment with virus.

Neutralization tests on undiluted sera taken 44 days after the last administration of virus were negative. Saline extracts of the isolated bowel segments and of the regional lymph nodes failed to inactivate the virus.

M48. An Investigation into the Mechanism of Adult Immunity against Poliomyelitis. MAURICE BRODIE AND WILLIAM H. PARK, Department of Bacteriology, New York University and Department of Health, City of New York.

Two hypotheses have been offered for explaining the relative insusceptibility of adults to the virus of poliomyelitis. The first of these maintains that a specific immunity develops, the result of exposure to the virus, while the second presupposes that the resistance develops irrespective of the etiological agent and is merely a physiological change occurring with age.

Accordingly the serums of a series of 14 monkeys, whose ages ranged between 15 months and 15 years, equivalent in the human scale to between 8 years and middle age, were tested against a minimal completely paralyzing dose of virus. None of the serums neutralized the virus. Moreover, two of the older and larger animals were unable to withstand, per kilo of weight, any more virus than younger and smaller monkeys. Thus the experimental animal, which has little or no opportunity for exposure to the virus, fails to develop immunity with growth.

On the other hand 27 out of 34 serums, obtained from adults, where exposure to the virus was likely, showed considerable neutralizing substance. Moreover those with a history of frequent exposure to the virus showed a slightly higher antibody average content than those who had no knowledge of contact. On the other hand a series of 32 children, whose ages ranged between 1 and 7 years and who had probably had insufficient exposure to the virus to develop immunity, failed to show except in a few instances, an appreciable amount of antiviral substance. Thus immunity as evidenced by the neutralizing power of the serum for the virus, seems to develop only where there is an opportunity for exposure to the virus. Additional evidence was obtained by the production in monkeys of antiviral substance, upon repeated intranasal instillation of virus into the nares of animals with cut olfactory nerves.

In keeping with the development of immunity from specific exposure to the virus, was the fact that the immunity was not related to that of diphtheria and so immunity to each was probably the result of its own nonspecific incitant. In a series of 30 children and 19 adults, the Schick

test and presence of poliomyelitis neutralizing substance did not correlate. Likewise, in a small series of 26 adults, 11 of whom were in the A group and 15 in the O group, the incidence of antiviral substance was approximately the same for either group.

In keeping with the epidemiological evidence of others our experiments suggest that this immunity developed only after prolonged exposure to the virus.

M49. Active Immunization of Experimental Animals and Children Against Poliomyelitis with Formalin Inactivated Virus Suspension. MAURICE BRODIE, Department of Bacteriology, New York University and Department of Health, City of New York.

In previous communications it was pointed out that definite immunity could be developed against the virus of poliomyelitis using virus rendered non-infective by formalin. However, the amount of formalin used produced considerable skin irritation. Since then it has been shown that virus suspension inactivated with either 0.2, 0.1 or 0.05 per cent formalin at incubator temperature, was also antigenic for monkeys, and at the same time, the two latter strengths gave practically no skin irritation. The majority of the animals showed both humoral immunity as tested by the neutralizing power of the serum for the virus of poliomyelitis, and tissue immunity, that is resistance to intracerebral inoculation of active virus.

The inactivated antigen produced no reaction whatsoever; neither symptoms, temperature rise nor cerebrospinal fluid changes developed upon repeated inoculations of large doses given both intracerebrally and intraperitoneally, each representing the equivalent of thousands of infective doses of living virus. Moreover during vaccination the animal suffered no untoward local or systemic reactions and so it was felt that the vaccine could be given to humans with perfect safety.

However, before doing so upon children, it was deemed advisable to try it upon ourselves to determine whether the vaccine produced any disagreeable local or general reactions. Accordingly, 6 volunteers from the Bureau of Laboratories, Department of Health, New York, were given 3-cc. doses of 10 per cent virus suspension, inactivated with 0.1 per cent formalin for 16 to 48 hours. These were given: three, 1 dose; two, 2 doses and the third, 3 doses. The second inoculation was given 11 days after the first, and the third, 8 days after the second. After inoculation there was some soreness at the site of injection, lasting but a few minutes and probably due to the formalin. Three of these injected

had some induration, which lasted but a few days and was not painful or uncomfortable. In no instance was there any systemic reaction.

The blood serums of all 6, obtained before immunization, were tested for antibody and the amount was determined by careful titration. A preliminary test was carried out upon the serums of the 3 who had more than one dose of vaccine and some increase in antibody was demonstrable.

The vaccine was then given to two series of children the first consisting of 12 and the second of 20 children, whose ages range between 1 and 7 years. The virus suspension used for preparation of the vaccine was cultured aerobically and anaerobically before it was treated with 0.1 per cent formalin for 12 to 16 hours, 12 hours being the time required to inactivate the virus. In the first series, 5 received a single dose of 5 cc., the others were given a second dose, either 11 or 13 days later. In the second series one half were given 5 cc. and the other half 2.5 cc. of antigen and half of each group received one and two doses, respectively. One to 2.5 cc., was given intracutaneously, the remainder subcutaneously. The material was injected into the skin of the abdominal wall.

The children were carefully observed for local and general reactions and temperatures were recorded 4 times daily. There was no apparent general reaction or discomfort and at no time any febrile manifestations that could be attributed to the vaccine. The local reaction was negligible, consisting only of some induration in those receiving the larger amounts intracutaneously. The first dose did not render the children sensitive to the second dose.

To determine the degree of immunity produced by the vaccine, the neutralizing power of each serum was tested before and 3 or 4 weeks after the administration of the vaccine. In each instance the antibody or antiviral content of the serum was determined quantitatively by estimating the number of minimal completely paralyzing (M.C.P.) doses the virus neutralized. The M.C.P. dose represents the smallest amount of virus-containing tissue that will produce a complete and rapid paralysis in a monkey of 2.5 to 4 kgm. within 13 days.

Almost all of the children showed, prior to immunization, a small amount of neutralizing substance. Up to the present only the first series have been completed and all of the children have shown an appreciable response to the antigen. The blood serums of 6 children neutralized between 100 and 200 and the other 6 between 200 and 600 additional infective doses of virus when examined 3 weeks after immunization. The immunity developed within a week.

M50. Avian Psittacosis. K. F. MEYER AND B. EDDIE, Hooper Foundation, University of California, San Francisco.

The course of psittacosis as observed for a period of 295 days among ricebirds and parrakeets in small cages and also in a large aviary was briefly as follows:

(1) Spontaneous psittacosis among ricebirds is a rapidly fatal disease. In exposure experiments the longest incubation time recorded was 98 days.

(2) The virus present in the organs is discharged in the urine, faecal droppings and nasal mucus.

(3) Parrakeets are less susceptible to psittacosis than ricebirds. Latent infections are quite common and of greatest importance in the epidemiology of human and avian psittacosis.

(4) Immature and unmasked parrakeets succumb more readily to the virus than the mature breeding birds.

(5) The latent infections in a flock of parrakeets may, within 6 to 8 months, be reduced to 1 to 2 per cent. These carriers are non-infectious. With the resumption of breeding operations after an elapse of from 6 to 8 months psittacosis will flare up again and parrot fever in young and even old birds will be noted.

M51. The Specific Behavior of Bacteriophage. PHILIP LEVINE AND A. W. FRISCH, Department of Pathology and Bacteriology, University of Wisconsin, Madison.

Recent observations made in this laboratory, namely, the specific reaction between phage and carbohydrate containing extracts of bacteria and the demonstration of qualitatively specific fractions in polyvalent phages, are in harmony with the theory that the specificity of bacteriophage and antibodies depend upon similar, if not identical, substances.

With the aid of the phage absorption technic employed, i.e., tests for residual phage in the presence of heat-killed absorbing organisms, several of the somatic factors in the *Salmonella* group could be identified. At the same time qualitatively specific differences within the suipestifer group were demonstrated since several strains, absorbing poorly from a certain anti-suipestifer phage, were found to absorb small quantities of a phage for paratyphosus B. Experiments were then made to determine whether or not these strains could also be characterized by specific antibodies. Sera were produced by injecting rabbits with boiled organisms of the several varieties selected in the tests with phage and cross

absorption experiments were performed. The results of these tests point to the existence of qualitative specific antigenic differences within the suipestifer group attributable to somatic (heat-stable) factors. It remains to be determined whether or not there is a complete correlation between the observations made with phage and those with antibodies.

M52. A Comparison of Streptococci from the Colon with Bargen's Organism. JOHN F. KESSEL, Los Angeles County Hospital and School of Medicine, University of Southern California.

Streptococci isolated from stools and from proctoscopic scrapings of patients exhibiting symptoms of colitis and of individuals with no colitis symptoms have been compared with 5 strains of streptococci commonly designated as Bargen's "diplo-streptococcus."

The streptococci observed have fallen into 10 different fermentative types when grown in media containing inulin, mannitol, salicin, lactose, saccharose and raffinose, respectively.

All the types compared in this study have produced methemoglobin on blood agar plates, have resisted a temperature of 60°C. for 30 minutes, and have exhibited similar morphologic and cultural characteristics. None has produced intestinal lesions when injected intravenously into rabbits, though kidney lesions have been a common result of such injections.

By the use of mannitol and salicin only, it is possible to refer each of these 10 types to one of the following species of Andrewes and Horder: *S. faecalis*, *S. mitis* or *S. salivarius*.

Agglutination reactions do not correlate to any appreciable degree with the 10 fermentative types listed. A more marked serologic relationship exists, however, between strains giving fermentation reactions of *S. faecalis*, *S. mitis* and *S. salivarius*, respectively.

No one type has been associated more frequently with colitis patients than with non-colitis patients.

Of the 5 strains of Bargen's organism compared, one was identical with our Type I which is also identical with *S. faecalis* of Andrewes and Horder; one with Type II which is a variant of *S. faecalis*; one is identical with our Type V, *S. mitis* of Andrewes and Horder, one with Type II, a variant of *S. mitis* and two with Type VIII, also a variant of *S. mitis*. Bargen's strains did not exhibit any apparent differences from our type in (1) the action of blood plates, (2) heat resistance qualities or (3) pathogenicity in rabbits.

It is, therefore, concluded that there is no bacteriologic justification

for regarding the streptococci described by Bargen as being essentially different from other types of alpha streptococci commonly recovered from the human alimentary tract.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. A Study of Methods for Determining the Numbers of Yeasts and Molds in Butter. E. H. PARFITT, Purdue University Agricultural Experiment Station, Lafayette, Ind.

Various media which have been used in determining the yeast and mold count of butter were compared, and it was found that potato dextrose agar was the most satisfactory in that it yielded the highest counts. The composition of potatoes, as would be influenced by potatoes grown under widely different conditions and stage of maturity, did not affect the count of yeasts and molds. No advantage was secured by adding ammonium chloride, dipotassium phosphate and calcium chloride to the basic medium.

The kind of acid used to acidulate the medium to pH 3.5 ± 0.1 was found to affect the growth of yeasts, lactic acid causing the greatest inhibition and tartaric acid the least. Lactic acid was neutralized by the growth of yeasts to a greater degree than was tartaric acid.

The maximum count was secured after 5 days incubation at 21–25°C., though the count secured after 3 days incubation at 21–25°C. closely approached the 5-day count.

The oxidation-reduction potential of the media within the limits studied, did not appear to be the limiting factor in the growth of yeasts and molds found in butter upon the media used.

A2. Study of the Lipolytic Enzymes of Three Specific Organisms. GEORGE SPITZER AND E. H. PARFITT, Purdue University Agricultural Experiment Station, Lafayette, Ind.

The lipolytic activity of the enzymes of *A. lipolyticum*, *Ps. mucidolens* and *Ps. fluorescens* were determined on an emulsion of milk fat and tributyrin. The effect of sodium chloride and acidity was also studied.

The lipolytic enzymes of the three organisms were less active on the alkaline side than on the acid side (pH 7.5 to 5.5). In the substrate at pH 7.0 there was definite inactivation of enzymatic lipolysis in the presence of 8.0 per cent sodium chloride and a much greater inactivation with 16.0 per cent.

The enzymes of the organism were obtained by growing the organisms

in peptonized milk for 7 days at 21°C., and then autolysing in the presence of toluol for 7 days. The autolysate was then added to an emulsion of milk fat and to an emulsion of tributyrin.

A3. The Bacteria Responsible for Ripeness in Emmental Cheese Milk. ARTHUR B. EREKSON AND W. DORNER, The Swiss Institute for Dairy Research, Liebefeld, Switzerland.

Ten representative samples of kettle milk from 8 different factories located near Bern, Switzerland, were studied to determine the types of organisms responsible for so-called ripeness.

Because of the unsatisfactory growth of some of the milk-ripening bacteria in plate cultures and on the surface of agar, a dilution method with transfers to tubes of sterile milk was employed. Selective incubation temperatures with one set of tubes at 30°C. and another at 48°C. were used to differentiate between *Strep. lactis* and *Strep. thermophilus*. Cultures from the highest dilutions were isolated and identified.

The rates of acid development in portions of kettle milk held at 30°, 37°, 48°C. and the Emmental cheese making temperatures were also determined as a check on the types of bacteria causing the first change in acidity.

The results showed that *Strep. thermophilus* was the predominant organism in 9 out of the 10 cases studied. In the exceptional case where *Strep. lactis* ranked first, the cheese was of inferior quality. Acid-proteolytic bacteria were present in significant numbers (600,000 per cubic centimeter) in one lot of milk from which very good cheese was made. Lactobacilli did not appear to be important although certain types related to *Thermobacterium lactis* (Orla-Jensen) were found growing in the milk after it had been held at 48°C. for 24 hours.

A4. The Disinfection of Beverage Glasses by Chlorine Preparations. W. L. MALLMANN AND E. D. DEVEREUX, Department of Bacteriology, Michigan State College, East Lansing.

A survey of places dispensing beverages revealed that glasses were inadequately cleansed and were not sterilized prior to use. The rims of supposedly clean sterile glasses were swabbed with sterile cotton swabs and the number of bacteria present was determined. Counts of 50,000 to 100,000 per glass were not uncommon. Few glasses were sterile. Many places were making no attempt to sterilize the glasses while others, although using chlorine preparations, were not obtaining the

desired results. Laboratory tests using *Staphylococcus aureus* were made to determine the minimum safety periods that would insure sterility. A procedure for proper disinfection is presented.

A5. An Investigation of the Sterility of Fish Tissues. B. E. PROCTOR AND J. T. R. NICKERSON, Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

Experiments were made to determine the presence of bacteria in fish muscle, fish blood and other tissues. Muscle samples were taken from haddock frozen on ship-board by carbon dioxide ice, while the blood samples were taken from the hearts of live fish within a few minutes after they were caught.

(1) Ninety-six sub-surface samples of fish muscle were removed aseptically with sterile gouges and transferred directly into fish agar and fish broth. After incubation at 20°C. for 168 hours 95 were found to be sterile.

Twenty-four muscle samples from the same fish were transferred to flasks of sterile dilution water, shaken mechanically for 15 minutes, and various dilution plates and tubes made therefrom. All plates and tubes from 20 of the samples were sterile after incubation. In the other 4 samples only occasional bacteria were found.

Samples taken from the gills, intestinal walls and skin showed the presence of bacteria in every case.

(2) Thirty-eight fish muscle samples were excised aseptically, placed in sterile flasks, stored at 0°, 5°, 10°, 15°, or 17°C. for 1 to 5 days and portions thereafter transferred to nutrient broth and nutrient agar plates. After incubation 34 were found to be sterile.

(3) One squirrel hake and two silver hake were caught off Gloucester. The fish were opened, using aseptic precautions, and blood was taken from the heart of the living fish within a few minutes of the time of capture. Transfers from the blood samples into solid and liquid media were held under both aerobic and anaerobic conditions. No organisms developed under anaerobic conditions. No aerobic growth was obtained with the exception that one plate contained one colony.

Of the 158 fish muscle samples examined, 149 or over 94 per cent were found to be sterile in the media used. Samples taken from the heart blood of three different fishes were found to be sterile in two instances, and in the third instance there is evidence which suggests that this sample also was sterile.

A6. Some Biochemical Changes Exhibited in Fish Muscle Stored at Different Temperatures under Normal Conditions and under Sterile Conditions. J. T. R. NICKERSON AND B. E. PROCTOR, Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

A series of biochemical determinations was made on samples of fish muscle excised from 8 haddock and stored at temperatures of 0°, 5° 10°, 15° and 17°C. These fish were frozen in the round immediately after taken from the water and kept below −17.8°C. until the start of the investigation.

Comparisons were made on two groups of samples, taken in each case from the same fish: (*a*) Muscle samples which were excised under aseptic conditions and stored in sterile flasks at one of the above temperatures for a definite time. (*b*) Muscle samples excised after the fish itself had been stored for the same time and at the same temperature.

Van Slyke amino-nitrogen determinations, pH, and the electrometric titrations by the method of Stansby and Lemon were carried out on 4 of the haddock. Formol titrations (Harris modification), ammonium nitrogen determinations, pH, and the electrometric titration were the methods used on the remainder.

The ammonium nitrogen values obtained showed a greater magnitude of change than the other constituents determined. This was especially evident in the (*b*) samples at the higher storage temperatures.

The pH of all samples was found to be between 6.55 and 6.95. The (*b*) samples showed a slight increase in pH on storage, whereas the (*a*) samples evidenced a tendency to rise in pH slightly with a subsequent decrease.

The amino-nitrogen values of (*a*) samples evidenced a slight rise, followed by a subsequent lowering. In the (*b*) samples, the same trends occurred, but were followed by a final increase in value. The formol titration values indicate somewhat similar trends to those exhibited by amino-nitrogen.

The electrometric titration method gave results which differed somewhat from those reported by others for fresh fish. The present work, however, was concerned only with fish which were frozen while fresh.

A7. The Relationship of Crystal Surface Contaminants to the Biological Quality of White Sugar. H. H. HALL AND L. H. JAMES, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

Substances, such as impurities, which adhere to the surface of sugar crystals and which stimulate the growth of yeast, influence the biological quality of white sugar. Also, thermophilic bacteria which are located mainly on or near the crystal surface impair the quality of white sugars.

A simple and effective method for the removal of these crystal contaminants is described.

A8. Electro-Silver Preservation of Fruit Juices. LAWRENCE H. JAMES AND E. A. BEAVENS, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

A brief résumé of the literature is given. The results obtained when apple cider and grape juice were seeded with bacteria and yeasts and treated with silver under the stimulus of a small electric current indicate that when sufficient silver is present fermentation may be prevented.

A9. A Study of Lactobacillus plantarum (Orla Jensen) Bergey. CARL S. PEDERSON, New York Agricultural Experiment Station, Geneva.

In the latest edition of Bergey's Manual the key to the genus *Lactobacillus* was revised so as to group certain organisms that apparently were similar. One of these groupings contained among several others, the species *Lactobacillus plantarum* Bergey, originally named *Streptobacterium plantarum* by Orla Jensen. Orla Jensen's description of this species is apparently quite broad for it is found that the 44 strains described are variable toward certain sugars, especially xylose, arabinose, and raffinose and somewhat variable in their growth temperature and in their heat resistance. Although the majority of strains form inactive lactic acid, a few form dextro lactic acid. The morphology is variable.

A study of the characters of several hundred cultures isolated from many sources has shown considerable variation between individual strains, but only a more or less natural variation when the entire group is considered. Failure to find any character which could be used to split the group definitely forces one to consider the group as a single species.

The following species are considered synonyms:

Bacillus cucumeris fermentatae Henneberg, *Bacillus wortmanni* Henneberg, *Bacillus listeri* Henneberg, *Bacillus aderholdi* Henneberg, *Bacillus maerckeri* Henneberg, *Bacillus leichmanni* II Henneberg, *Bacillus beijerincki* Henneberg, *Bacillus pabuli acidi* Weiss, *Lactobacillus pentosus* Fred, Peterson and Anderson, *Lactobacillus arabinosus* Fred, Peterson and Anderson, and *Bacterium busaeasiaticae* Tschekan.

A10. The Carotenoid Pigments of Several Bacterial Species. MARY A. Ingraham, University of Wisconsin, Madison.

The pigments of *Mycobacterium phlei*, *Corynebacterium carotenum* and a red diphtheroid were separated by partition between various solvents, and by differential adsorption on columns of magnesium oxide and calcium carbonate. Identification of the carotenoids present was made spectroscopically, and their identity was confirmed by observing their behavior in adsorption columns after mixing them with known pigments obtained from other sources.

Myco. phlei contained α-carotene, β-carotene, cryptoxanthene, lutein and zeaxanthene. α-carotene, β-carotene, cryptoxanthene and zeaxanthene were isolated from *C. carotenum*. The color of the red diphtheroid was largely due to the presence of lycopene, but β-carotene and zeaxanthene were also present.

Of these pigments, three (α-carotene, β-carotene and cryptoxanthene) are biologically active as sources of vitamin A. The activity of β-carotene is twice that of the other two compounds. The theoretical vitamin A activity of the bacterial cells, calculated from quantitative analyses of these pigments, agreed very closely with the experimental results obtained when the cells were fed to rats on a vitamin A deficient diet.

The proportion of the various pigments, as well as their absolute amounts, varied with age and cultural conditions.

A11. Studies on the Classification and Differentiation of the Rhizobia. W. B. SARLES, J. C. MCCAFFREY AND M. N. MICKELSON, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

Strains of several species of *Rhizobium* of varying ability to benefit their respective host plants were studied in an attempt to find new characteristics of value in their classification.

On sodium selenite-yeast water agar, strains of the fast-growing species became red in 4 days; *Rhizobium japonicum* and *Rhizobium lupini* strains grew slowly and produced little or no red color. The cowpea *Rhizobium* strains were intermediate in their action. *B. radiobacter* became red in 4 days.

Clark and Lubs medium supported growth of the cowpea *Rhizobium* and of *B. radiobacter*; none of the other rhizobia grew in this medium. Neither of the species which grew gave a positive methyl red test or Voges-Proskauer reaction.

Strains of the cowpea *Rhizobium* grew in the sodium citrate medium

commonly employed in water analysis. None of the other rhizobia grew in this medium; *B. radiobacter* showed very limited growth.

On calcium-glycerol-phosphate agar, *B. radiobacter* produced a heavy, brownish growth and a browning of the medium. None of the rhizobia tested produced either a brown growth or a browning of the medium.

By altering the composition of the media used, the ability of the rhizobia to liquefy gelatin and reduce nitrates could be varied greatly.

An attempt was made to determine the possible relation between rate of growth and ability to benefit the host plant of strains of *Rhizobium japonicum*. As a rule, beneficial strains of this group grew rapidly at 25°C. in a liquid medium, but not all fast-growing strains were beneficial.

A12. The Effects of Storage on Rhizobia. J. J. Reid, E. B. Fred, and I. L. Baldwin, University of Wisconsin, Madison.

The study of rhizobia at the University of Wisconsin, covering a period of more than 20 years, has resulted in an accumulation of cultures together with data pertaining to them that has made this series of investigations possible. Twelve years ago 40 strains of rhizobia were dried down in sterilized soil and placed at room temperature in Freudenreich flasks stoppered with glass wool. In 1934 the flasks were opened and an attempt was made to recover the organisms. It was found that with the exception of strains of *Rhizobium leguminosarum* viability was good. The strains recovered were compared culturally and in the greenhouse with the corresponding cultures carried during the 12 year interval on yeast water mannitol mineral salts agar with frequent transfer. Only in the case of *Rhizobium japonicum* had any variation appeared—in all other groups the cultural characteristics and host plant relationships of the strains on agar were identical with the corresponding strains recovered from the soil after a resting period of 12 years.

Two years ago pure cultures of *Rhizobium meliloti* and *Rhizobium japonicum* were dried in milk in vacuo at a low temperature and the cultures sealed in glass vials. These have been held under various conditions and examined at intervals. It was found that storage at temperatures higher than 10°C. resulted in rapid decline in the number of viable organisms. The dried cultures were less effective in their ability to benefit the host plant than cultures of the same strain carried on agar. The infectiveness of *Rhizobium japonicum* decreased at a greater rate than did the viability.

Pure cultures of *Rhizobium meliloti* and *Rhizobium japonicum* were grown on yeast water mannitol mineral salts agar and held at tempera-

tures of 37°, 25° and 10°C. It was found that *Rhizobium japonicum* was much more susceptible to high storage temperatures than was *Rhizobium meliloti*. Little difference was found in cultures stored at 25° and 10°C. The viability was good at these temperatures for a period of 4 months but at the end of 12 months the count had declined to 5 per cent of its initial value.

A13. The Respiration and Growth of Rhizobium As Influenced by the Reaction of the Medium. D. W. THORNE AND R. H. WALKER, Iowa State College, Ames.

The effect of reaction on growth and respiration of *Rh. meliloti* was studied using Warburg respirometers. The organisms were suspended in nutrient media buffered at different reactions, ranging from pH 3.5 to 9.0, with phosphate salts. The reactions of the media were determined at the beginning and end of each experiment with either the quinhydrone or glass electrodes. Oxygen consumption was measured as the criterion of growth and respiration.

This species was found to have maximum respiration and growth rates near neutrality with fairly constant activity between pH 6.0 and 7.8. Beyond these reactions the rates decreased rapidly to a minimum on the acid side at pH 3.6 and on the alkaline side at pH 9.0.

Similar studies are being conducted with other species of this genus.

A14. Moisture Control in Studies of Symbiosis Between Rhizobia and Legume Plants. ALVIN W. HOFER, New York Agricultural Experiment Station, Geneva.

In studies of the effect of various strains of rhizobia upon legume plants, careful attention must be given to all factors that might influence the results. The moisture supply, however, has been difficult to control, and since it is capable of great fluctuation, especially in conditions most favorable for growth of plants, some method is needed for its regulation. The apparatus described here has been designed to satisfy this need.

A Mariotte bottle (Science, **80**: 100) is connected by a rubber tube to a "T" fitted into a porcelain auto-irrigator cone set into the sand. The connecting tube is fitted with a downward detour by means of two "Y's", in such a way that it can be closed off to allow the use of mercury for setting up any desired resistance against the capillary pull of the sand. When the "T" above the pot is extended by means of a short rubber tube, it may be closed with a pinch-clamp. This may be opened to

allow air to be blown out of the system. Use of the apparatus is facilitated by use of an additional tube extending a short distance into the Mariotte bottle, to allow escape of air when filling. This simple device provides accurate regulation of moisture in the pots. An advantage of the apparatus is the fact that there is nothing in it which cannot be sterilized by steam.

A15. Quantitative Studies of Three Species of Rhizobium in Soils of Different Fertilizer Treatment. A. G. LOCHHEAD AND R. H. THEXTON, Division of Bacteriology, Central Experimental Farm, Ottawa, Canada.

The numbers of *Rhizobium trifolii*, *Rh. leguminosarum* and *Rh. meliloti* were determined at three to four-week intervals for three years in three areas which had been receiving for 20 years no fertilizer, manure, and artificial fertilizer, respectively. *Rh. trifolii*, the only species with host plant in the rotation, was present in much greater numbers than the others, not only immediately following clover, but in later years when numbers had ceased to decline. Whereas quantitative differences between the soils were slight in the case of *Rh. trifolii*, the two fertilized soils harbored noticeably higher numbers of *Rh. leguminosarum* (19 and 20 times) and *Rh. meliloti* (5 and 11 times) than did the unfertilized area. The reverse condition held with *Azotobacter* where the numbers of colonies were consistently higher in the unfertilized than in the fertilized areas. Freezing of the soil for approximately three months each year did not have any pronounced effect on the numbers of the nitrogen-fixing organisms studied.

A16. The Numbers of Rhizobium meliloti and Rhizobium trifolii in Soils as Affected by Soil Management Practices. R. H. WALKER AND P. E. BROWN, Iowa State College, Ames.

Wilson's modification of the dilution method was used in determining the approximate numbers of *Rhizobium meliloti* and *Rhizobium trifolii* in variously treated Iowa soils.

It was found that, in general, the number of these root-nodule bacteria in soils depends upon the previous cropping history of the land, and also upon the fertilizer treatments that have been made to the soil.

Larger numbers of both species were found in the soil of cultivated fields than in the soils of adjoining virgin prairie sod. There were also larger numbers of these organisms in soils where a three-year crop rotation, including a mixture of alfalfa and red clover, was followed

than in soils where a two-year cropping system without legumes was practiced.

In general, the application of manure, limestone and phosphate fertilizers to soils served to increase to a large extent the numbers of both the alfalfa and red clover root-nodule bacteria. Of the soils examined those that had been regularly treated with manure contained from 10 to 100 times as many of these species as adjoining plots of untreated soils. Applications of ground limestone in addition to the manure to this soil which had an original pH value of about 5.5 increased the numbers of these bacteria another ten-fold. Phosphate fertilization in addition to the other treatments gave a still further increase in numbers.

Similarly treated soils upon which both alfalfa and red clover were grown in the rotation contained approximately the same number of alfalfa and red clover root-nodule bacteria.

A17. Oxidation-Reduction Potentials and the Activity of Marine Nitrifiers. CLAUDE E. ZOBELL, Scripps Institution of Oceanography, La Jolla, Calif.

Pure cultures of nitrifiers which oxidize ammonia to nitrites have been isolated from marine bottom deposits. The oxidation-reduction potentials of such cultures have been determined electrometrically, using Pt electrodes, a sensitive galvanometer and potentiometer, and a saturated KCl calomel reference cell. Potential-time curves during the growth of these nitrifiers in ammoniacal sea water solutions were almost straight lines, or in some cases, when the initial oxidation-reduction potential was low, there was a positive drift, nitrites beginning to appear when the potential reached E_h 0.25 to 0.35 volt at pH 8.0 and 20°C. By adjusting the potential with poising substances in concentrations which are otherwise inert, the optimum oxidation-reduction potential for nitrification was found to be E_h 0.30 to 0.55 volts, multiplication without nitrite formation occurring at somewhat lower potentials. Nitrification occurs in poorly aerated media or in media rich in organic matter provided the oxidation-reduction potential is favorable.

While a few nitrifiers have been demonstrated in marine bottom deposits having an oxidation-reduction potential as low as E_h -0.1 to -0.3, it is questionable whether they are active in such an environment. Frequently there is no evidence for the presence of nitrifiers when several grams of mud are inoculated into the test medium, although much smaller inocula yield positive results, this being attributed to the unfavorable poising capacity of large quantities of mud.

A18. Nitrogen Transformations of Ammonium Sulphocyanate, a Weed Eradicator, in the Soil. C. E. SKINNER AND ALLAN SANDHOFF, University of Minnesota, Minneapolis.

Ammonium sulphocyanate in quantities comparable to the amounts used in agricultural practice increased the number of bacteria, as determined by the poured plate method, in all cases. When used in larger amounts, the numbers were decreased. A number of bacteria were isolated which used NH_4CNS and NaCNS as the only N source in glucose-mineral liquid medium. Neither algae nor protozoa were depressed when NH_4CNS was used in large doses in the soil. When used in small doses, only the NH_3N of the NH_4CNS was recovered as ammoniacal or nitrate N after long incubation periods. A small amount of inorganic N, above the uninoculated control, was recovered from soil to which NaCNS was added. Partial sterilization effects were considered to be ruled out. It is concluded that only one-half of the nitrogen of NH_4CNS can be considered as an available fertilizer.

A19. The Effects of Microbiological Activity During the Aging of Some Moist Ceramic Materials. DUDLEY P. GLICK, Colorado State College.

The primary object of these investigations, begun in 1931 at Ohio State University, was to determine the validity of the "Bacterial Theory"—an hypothesis advanced by some ceramists to account for the increase in workability and strength in certain ceramic bodies pursuant upon their aging prior to their being molded and fired as ceramic ware.

Freshly prepared clay bodies were divided into duplicate series of 1000-gram samples. One series was sterilized by autoclaving. Half of the samples of each series were incubated at approximately 5°C., the remainder at approximately 28°C. Samples of the sterile series were tested immediately following autoclaving and samples of all bodies were tested at the end of one week, 4 weeks and 8 weeks. The tests made were: Atterberg plastic index, dry transverse strength, water of plasticity and dry shrinkage.

The data secured indicate (1) that the quality of the unsterilized clays improved as much in one week as that of the sterile bodies improved in two months; (2) that an incubation temperature of 28°C. is more favorable than 5°C. for the development of plastic properties in the raw samples whereas the temperature of incubation exerts no effect upon the sterile samples; (3) that up to a period of one month the

properties of unsterile clays are improved almost proportionately with the time of aging and that during the second month the clays continue to improve but at a lower rate; (4) that bacterial activity was greatest in ball clay having an organic matter content of 5 per cent to 6 per cent; and (5) that other influences are at work during aging that tend to increase the workability of clay but that these effects are slight when compared to the effects of microbiological activity.

A freshly prepared porcelain body was set up into raw and sterile series and 1000-gram samples of each series were treated as follows: (1) untreated, (2) 10 per cent (by weight) of horse urine added, (3) 10 per cent manure water, (4) 1 per cent tannic acid, (5) 1 per cent peptone, and (6) 1 per cent ammonium nitrate. All samples were aged at room temperature (approximately 25°C.) for a period of 12 months at which time they were tested as above and plate counts of the raw samples were made upon beef-infusion agar, pH 7.4, and upon clay-extract agar, pH 7.8. The greatest increases in plasticity of the raw samples as compared with the sterile samples were observed in the untreated samples and in those to which peptone had been added. The phenomena of thixotropy and greatly increased dry transverse strength resulting from the addition of tannic acid were diminished by about 40 per cent as the result of bacterial activity. The plate counts upon beef-infusion agar ranged from 3.1×10^8 in the untreated samples to 1.4×10^{11} in the tannic acid samples and upon the clay-extract agar varied between 3.0×10^7 in the untreated samples and 1.8×10^{10} in the samples containing manure water.

Aged bodies secured from potteries were examined bacteriologically and showed in general, the bacterial flora of the water supply used in the preparation of the clay mixtures. *Achromobacter* sp. predominated.

A20. The Microbiology of the Upper Air. II. BERNARD E. PROCTOR, Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge.

Collections of microörganisms present at various altitudes in the upper air were made during the course of 30 airplane flights in the vicinity of Boston. A record of the dust particles and pollen collected simultaneously was also made. The collection apparatus consisted of a sterilized metal container, within which were oil-treated lens-paper filters. The container was connected by tubing to an air inlet above the roof of the plane and air was drawn through the filters by means of a Venturi. The laboratory technique used enabled observations of only

those types of microörganisms capable of aerobic growth on standard nutrient agar.

Bacteria and molds were found to be present at altitudes greater than 20,000 feet in several flights. Lower altitudes were found to have microorganisms present in varying numbers. A comparison of dust particles and microörganisms obtained showed the dust particles to be much more numerous than bacteria at all altitudes. Certain of the microorganisms in these collections have been isolated and identified.

Observations made from collections on May 11, 1934, when a severe dust storm originating in the drought areas passed over Boston are also included and show great increases in bacteria.

A21. Study of the Chemical Mechanism of Anaerobic Methane Production.
A. M. Buswell, University of Illinois, Urbana.

A study has been made of the anaerobic fermentation of 91 pure substances, including carbohydrates, alcohols, acids, aldehydes and ketones in both the aliphatic and aromatic series. It has been shown that the fermentation is an anaerobic oxidation-reduction reaction involving water and catalyzed by bacteria. Carbon dioxide, methane, and hydrogen appear to be primary products, the hydrogen combining with a portion of the carbon dioxide to give additional methane and water. Organic acids resulting from alpha oxidation are indicated to be intermediates in the reaction. Thermodynamic calculations show that if water is the only source of oxygen besides that in the compound undergoing fermentation, carbon dioxide and methane are the end-products that might be expected since the greatest decrease in free energy occurs with their formation. The reaction follows a simple equation in which the number of molecules of water necessary for the reaction and the amounts of carbon dioxide and methane produced bear a mathematical relation to the formula of the compound undergoing fermentation.

The progressive changes in composition of gases evolved during fermentation have been used as a means for determining the course of the reaction. This method is advantageous since relatively small weights of material can be determined with considerable accuracy and it is not necessary to withdraw a sample of the fermentation mixture for analysis, or at most a very small sample will suffice. Application of the method to the fermentation of dextrose, levulose, sucrose, and starch indicates that aliphatic acids resembling butyric are first formed in the fermentation and later undergo decomposition to carbon dioxide and

methane. The amounts of hydrogen produced from different carbohydrates indicate that there is some difference between the manner of fermentation of dextrose and levulose, as compared with sucrose and starch.

A22. The Carbohydrate-Nitrogen Relationship in Symbiotic Nitrogen Fixation. P. W. WILSON, University of Wisconsin, Madison.

Previous work by the author and by others has emphasized the importance of the carbohydrate supply in conditioning many aspects of symbiotic nitrogen fixation in leguminous plants. Further experiments indicate that this explanation is too limited in its implications and leads to contradictions when applied to plants grown in certain environments. Evidence from many physiological and physical-chemical types of experiments makes it apparent that the important factor is not carbohydrate supply alone but the carbohydrate-nitrogen relationship obtaining in inoculated plants. The experiments include investigation of the following: effect of pressure of gases (CO_2, O_2, N_2), influence of combined nitrogen, effect of light intensity and day length, strain variation, effect of time of inoculation, and combination of these factors.

For purpose of discussion, 5 classes of plants are differentiated:

1. *Low carbohydrate—low nitrogen.* Invasion of plant, development of nodule and nitrogen fixation decidedly restricted.

2. *Medium carbohydrate—low nitrogen.* Invasion of plant, etc., restricted. (This is the normal type of growth in nitrogen-poor substrate under average light conditions and normal CO_2 supply.)

3. *High carbohydrate—low nitrogen.* Favors invasion of plant but is unfavorable to development of nodules and to nitrogen fixation. Between classes 2 and 3 is a region in which these three functions reach a maximum.

4. *Low carbohydrate—high nitrogen.* Inhibits invasion of plant, development of nodule and nitrogen fixation.

5. *High carbohydrate—high nitrogen.* Favors development of nodules and fixation of nitrogen as compared with class 4.

The hypothesis affords a unified biochemical explanation of many functions of the symbiotic nitrogen fixation process which previously were restricted to empirical description, e.g., effect of CO_2, strain of organism, and combined nitrogen and of the nitrogen hunger stage.

A23. The Relationships Between the Biological Activities and Certain Chemical Constituents of Muck. HAROLD W. BATCHELOR AND

ALFRED H. PASCHALL, Ohio Agricultural Experiment Station, Wooster.

Eight samples of muck representing a wide range of natural vegetation were analysed for calcium, nitrogen, ash, organic carbon, and several organic fractions.

The biological activities of these samples as indicated by carbon dioxide production and ammonia and nitrate nitrogen accumulation were determined. In the untreated samples the activities as measured by carbon dioxide production could not be correlated with the above analyses. In the case of the samples treated with cellulose there was an apparent relationship between the evolution of carbon dioxide and the carbon-nitrogen ratio, the samples with a ratio of less than 13 having the higher activities, whereas those with a ratio of more than 13 having increased activities with increased ratios.

The activities as indicated by ammonia accumulation failed to show any relation with the factors studied. However, the content of hemicellulose and cellulose did appear to have a relation to the length of time requiredfor the untreated samples to recover from a period of dormancy.

A24. A Study of the Microbiological Decomposition of the Constituents of Alfalfa Hay and its Application to Spontaneous Heating. MAX PHILLIPS, M. J. GOSS, E. A. BEAVENS AND L. H. JAMES, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

Previous studies on the spontaneous heating and ignition of hays and other agricultural products have shown the necessity for a more thorough knowledge of the fate of the various chemical constituents resulting from the fermentation and heating during storage. A laboratory study was made on the microbiological decomposition of first and third cutting alfalfa hay fermented under aerobic conditions at incubation temperatures of 30° and 55°C., and under anaerobic conditions at 30°C.

The greatest decomposition took place in the alcohol-benzene extractives, hot and cold water extractives, the uronic acid anhydrides (the pectins and the polyuronides fraction generally), crude protein (N × 6.25), and the methoxyl in the unextracted plant material. The pentosans decomposed somewhat more slowly, and the rate of decomposition of the Cross and Bevan cellulose and of the "pure" cellulose was still less. In general, lignin was found to be the most resistant of all the major plant constituents, although substantial losses of lignin were obtained under both aerobic and anaerobic conditions. Results indi-

cate that the third cutting material decomposed somewhat more rapidly than that of the first cutting.

A theory on the possible rôle of unsaturated lignin in the spontaneous heating and ignition of plant materials is discussed.

A25. Some Bacteriological and Chemical Aspects of A. I. V. Silage. W. H. Peterson, L. S. McClung and H. R. Bird, University of Wisconsin, Madison.

Green alfalfa was chopped, acidified with a 2 N solution of mineral acids (HCl and H_2SO_4) to pH 3.5 (90 liters 2 N acid per ton of forage) and packed tightly into wooden barrels, which were then headed. At intervals of 7–10 days a barrel was opened, and examined for numbers and types of bacteria and for acids and changes in the forms of nitrogen. Platings were made on glucose yeast water agar incubated under aerobic and anaerobic conditions. Under aerobic conditions the counts (millions per gram of silage) for the several barrels were successively 190, 160, 66, 9. Approximately the same numbers of bacteria were found in the anaerobic series. No yeasts, molds, or common anaerobes were found. Colonies picked from plates were inoculated into litmus milk and brought about a slow reduction of the litmus and curdling of the milk. These cultures were predominantly non-sporeforming short rods, which were Gram positive and catalase negative.

The chemical analyses on barrel No. 2 showed the production of both volatile (2.72 per cent dry basis) and non-volatile acid (2.20 per cent) and the hydrolysis of the nitrogen constituents. The silage contained about twice as much water-soluble nitrogen (1.41 per cent) and amino nitrogen (0.80 per cent) as the green forage. Ammonia nitrogen also increased and represented about 6 per cent of the total nitrogen (2.65 per cent).

A26. Dissimilation of Glucose by Propionic Acid Bacteria. H. G. Wood, R. W. Stone and C. H. Werkman, Department of Bacteriology, Iowa State College, Ames.

The succinic acid formed by propionic acid bacteria originates according to van Niel from the yeast extract proteins and not from carbohydrates. Virtanen as well as Foote, Fred and Peterson, have reported large yields and suggest the carbohydrate source of the acid. Solution of the problem has been attempted by complete and serial analysis of the dissimilation of glucose by several species of propionic acid bacteria under different conditions of culture. Our investigations support the

following conclusions: (1) Schemes for the propionic acid fermentation previously proposed, are not inclusive enough to accommodate the data obtained. A more flexible and complete scheme is required. (2) Certain cultures form succinic acid in glucose fermentations synthetically from acetic acid. (3) Succinic acid is decomposed as an intermediate metabolite in the dissimilation of glucose to propionic acid and CO_2. (4) 4- and 2-carbon cleavage of glucose as proposed by Virtanen has been established in fermentations with a number of propionic acid bacteria. (5) The mechanism of the propionic acid fermentation varies with the environmental conditions and with the species. (6) Evidence from propionic acid fermentations supports the intermediate occurrence of methylglyoxal in carbohydate metabolism.

Results are correlated in a single scheme of fermentation and the experimental data are shown to fit this scheme.

A27. Intermediate Behavior of Succinic and Acetic Acids in the Propionic Fermentation. C. ERB, H. G. WOOD, R. W. STONE AND C. H. WERKMAN, Department of Bacteriology, Iowa State College, Ames.

Wood and co-workers have secured evidence by fermentation of glucose with proliferating propionic acid bacteria that succinic acid is formed from acetic acid by synthesis and in turn is decomposed to propionic acid and CO_2. Additional evidence supporting this intermediate behavior of acetic and succinic acids has been obtained by use of the Barcroft-Warburg technic. Resting cells in the presence of air dissimilated acetic acid with an uptake of oxygen and formation of CO_2. A dehydrogenation of two molecules of acetic acid to form succinic acid and a breakdown of the latter with the formation of CO_2 would yield such results. Succinic acid was found to be decomposed by *Propionibacterium* anaerobically; CO_2 was formed and in the presence of air oxygen was taken up. This oxygen uptake suggests formation of fumaric acid. It may be dissimilated to CO_2 and pyruvic acid according to the Thunberg series of reactions. If succinic acid is dissimilated according to this series of reactions one would not expect it to be decomposed anaerobically in the absence of hydrogen acceptors. The anaerobic decomposition may be explained, however, by assuming that hydrogen acceptors are formed in the endogenous fermentation of the bacteria.

Theoretically the formation of propionic acid and CO_2 from succinic acid would proceed without additional hydrogen acceptors after the

first molecule of pyruvic acid was formed. It is not necessary, however, to assume a Thunberg series of reactions. The propionic acid bacteria activate propionic acid in the presence of air and utilize oxygen. It is thus possible to have an oxygen uptake following the direct decarboxylation of succinic acid. Evidence for the Thunberg reaction series is not conclusive. However, acetic and succinic acids are shown to be activated.

A28. Oxidation-Reduction Potential Studies on Propionic Acid Bacteria. R. W. Stone, H. G. Wood, C. Erb and C. H. Werkman, Department of Bacteriology, Iowa State College, Ames.

The methylene blue technic with resting cells has been used in the determination of possible intermediate steps in the dissimilation of glucose by propionic acid bacteria. Wood and co-workers have presented evidence that these organisms convert acetic acid to succinic acid in the presence of suitable hydrogen acceptors. Additional evidence is presented that succinic acid is broken down to propionic acid and CO_2. Oxidation-reduction potentials of a reversible dye system in the presence of one of the substrates in question and resting cells were measured potentiometrically. The potentials were observed over a pH range of 5.5–7.0, maintained by phosphate buffers. Sodium acetate was found to act as a hydrogen donator to methylene blue in the presence of resting cells in the pH range studied. Sodium propionate behaved in a similar manner. The acetate ion is able to donate hydrogen to nitrates at pH 5.5–6.0. Higher fatty acids, butyric and valeric, did not show this activity; however, formic acid gives some evidence of ability to act as a hydrogen donator. In connection with the breakdown of succinic acid, evidence was obtained showing that succinic, fumaric and malic acids all are able to donate hydrogen to methylene blue. Hydrogen donation was shown at pH from 5.5–7.0 and with o-chlorophenol indophenol as well as with methylene blue. The oxidation-reduction potential-time curves for malic and fumaric acid follow the same course, indicating a close similarity in the behavior of these two acids.

A29. Products from the Fermentation of Glucose and Arabinose by Butyric Acid Anaerobes. A. F. Langlykke and E. B. Fred, University of Wisconsin, Madison.

Forty-nine cultures of butyric acid-forming anaerobes were studied with respect to the products formed in the fermentation of glucose and arabinose in a yeast water medium. On the basis of the analytical

results the cultures could be grouped according to the presence or absence of isopropyl alcohol.

Of 8 cultures which fermented glucose vigorously and produced isopropyl alcohol as one of the products, 3 produced both acetone and isopropyl alcohol in varying proportions. The majority of the cultures fell into the group producing acetone and no isopropyl alcohol, and this group included a large number of cultures which caused only weak fermentation of the glucose.

In general, poor fermentation of the glucose and low production of neutral products was accompanied by an increased acid production, as might be expected. In this situation ethyl alcohol made up an abnormally high percentage of the neutral products, probably because of greater ease of formation.

Among the neutral products of those cultures which produced isopropyl alcohol there was a lower percentage of butyl alcohol than was the case with those cultures which produced acetone and no isopropyl alcohol. Fermentation of arabinose was generally less complete than the fermentation of glucose and was featured by a higher acid production and a lower yield of neutral volatile products.

A30. The Effect of Asparagin and Related Compounds on Certain Butyric Acid Fermentations. E. L. TATUM AND W. H. PETERSON, University of Wisconsin, Madison.

The stimulative effect of potato and other vegetable extracts on the fermentation of starch and the production of butyl alcohol by certain butyric acid forming bacteria is due to the asparagin contained in these extracts. L-asparagin has been isolated from potato extract and identified chemically. This isolated asparagin stimulates the fermentations to the same extent and in the same way as the original extracts, *viz.*, it increases the fermentation of starch and the production of butyl alcohol ten fold over that in unsupplemented corn-mash. Three samples of commercial asparagin had the same effect.

Aspartic and glutamic acids are just as effective as asparagin in bringing about this stimulation.

Of related compounds, ammonium malate can replace asparagin or aspartic acid to some extent, and ammonium succinate can replace them to a lesser extent. Ammonium tartrate and inorganic ammonium salts have practically no effect on the fermentation.

No amino acids other than the dibasic acids have any effect, and since compounds related to the dibasic amino acids are somewhat effective, it

is concluded that the presence of a dibasic amino acid or a compound which may be transformed into such an acid by the organism is essential for a vigorous fermentation of starch and solvent production by certain strains of butyric acid-forming bacteria.

A31. The Morphology of Bacteria Causing Reddening of Salted Hides. L. S. STUART, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

Additional morphological data have been compiled on the causative organisms which were reported in U. S. D. A. Tech. Bull. 383. Studies of growths on salt-saturated agars and on dextrose agar without added salt reveal definite likenesses to *Clathrocystis roseo-persicina* Cohn (1875) and *Myxococcus rubescens* Thaxter (1892). These observations lend support to Thaxter's belief that his *Myxococcus* and Schroeter's *Cystobacter* (1886) were identical.

This organism is described as a peculiar spreading growth manifested by crateriform indentations of the agar with which no well developed stainable bacterial cells could be associated.

The organism is heterotrophic, since it grows in both organic and inorganic substrates. It produces hydrogen sulphide from a basal nutrient solution containing free sulphur. The addition to this basal nutrient solution of asparagin, bactopeptone, egg albumin, gelatine, casein, dextrose, levulose, sucrose, starch and cellulose, respectively, as enrichment nutrients resulted in the development of a large variety of vegetative cell types, ranging from coccoids and oval-shaped rods to club-shaped rods, long straight-sided rods, thread forms and granulated thread forms, depending upon the specific nutrient added.

A32. The Production of Lipolytic and Depilating Enzymes by the Aspergillus Flavus-Oryzae Group. L. S. STUART, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington, D. C.

During the course of investigations on possible methods for removing fat from greasy cattle hides, 36 strains of *Aspergillus flavus-oryzae* were examined for their ability to produce lipase. As grown on butter agar 25 of the 36 strains gave positive tests by the copper soap formation method of Berry. On beef-tallow agar 34 of the 36 strains gave positive tests by the same method. As demonstrated by increases in the titrable acidity of beef-tallow after digestion by macerated mycelia, using a modification of the method which Willstätter, Waldschmidt-Leitz, and

Mennen used in studying pancreatic lipase, all of the 36 strains studied showed some ability to produce lipase. However, the relative amounts produced varied widely with the different strains.

Calculated from results obtained with olive oil digestions on dry mycelial weight basis the most active lipolytic strain of *Aspergillus flavus-oryzae* produced only 0.0005 Willstätter lipase units per mgm. as compared to 0.0027 units for the same quantity of dry pancreatic tissue.

Although 9 of the 36 strains produced enzymes capable of completely loosening hair from salt cured soaked calfskin and 6 others loosened the hair slightly, no correlation is shown between the abilities of the various strains to produce lipolytic and depilating enzymes.

A33. Factors Influencing Growth and Sterol Production of Aspergillus fischeri. P. R. Wenck, H. C. Greene, and E. B. Fred, University of Wisconsin, Madison.

The growth and sterol production of 6 strains of *Aspergillus fischeri*, Wehmer, and of 8 single spore cultures from one strain of the same mold, have been studied under identical conditions. Both growth and sterol production varied with the strain and with the different single spore cultures. Cultures yielding large quantities of mycelium produced a lower percentage-content of sterol than those yielding small quantities of mycelium. In repeated trials the results for each mold varied; these variations averaged 5.1 per cent for growth and 9.8 per cent for sterol production when single spore cultures were used, and 25.2 per cent and 17.5 per cent, respectively, when the parent culture was used as inoculum.

Both growth and sterol production varied widely according to the composition of the medium. Increasing the glucose concentration slightly increased the percentage of sterol, whereas increasing the urea concentration decreased it; increasing both glucose and urea concentrations in the same ratio held the percentage of the sterol nearly constant. The types of carbon source and nitrogen source were important factors influencing the yield of mycelium and sterol. The best carbon source was glucose; the best nitrogen source, urea. The concentration of KH_2PO_4 had no effect except upon the rate of growth. The optimum initial pH of the medium depended largely upon the constituents of the medium.

The percentage of sterol increased with the time of incubation; sterol was formed even after complete utilization of the sugar in the medium and after the mycelium began to autolyze.

The optimum temperature for both growth and sterol production was 37°C.

A34. The Proteinase and Aminopolypeptidase of Aspergillus parasiticus. M. J. JOHNSON AND W. H. PETERSON, University of Wisconsin, Madison.

The proteolytic system of *A. parasiticus* consists of at least one proteinase, carboxypolypeptidase and dipeptidase, and at least two aminopolypeptidases. One of the aminopolypeptidases may be obtained substantially free from other enzymes. This peptidase is similar to intestinal aminopolypeptidase in that it does not attack dipeptides, except decarboxylated dipeptides, and does not attack peptides in which the free amino group is methylated or replaced by a bromine atom. Triglycine, which is readily hydrolyzed by purified intestinal aminopolypeptidase, is not attacked by this mold aminopolypeptidase. There exists in mold a second aminopolypeptidase which does hydrolyze triglycine. It is not known whether this glycyl peptidase also attacks leucyl diglycine. Glycyl leucyl glycine is apparently hydrolyzed by both enzymes.

The proteinase of mold tissue is apparently of the tryptic type. In its hydrolysis of gelatin, only 0.2 per cent of the peptide linkages are split before the resulting product begins to be attacked by peptidases. In gelatin hydrolysis, a large fall in viscosity at the beginning of digestion is accompanied by very little increase in NH_2-N.

SERENDIPITY[1]

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Nowadays, I suppose no one reads Horace Walpole. Sixteen volumes of his letters rest patiently on the shelves of the library. If you pick up Volume III and turn to page 204, you will find one of his chatty letters[2] to Horace Mann, in which he playfully boasts that he has a talisman by which he can find anything he wants by dipping for it. Then he tells how he discovered in an old book on Venetian arms the origin of the badge at the top of the Medici coat-of-arms. He writes:

> This discovery, indeed, is almost of that kind which I call *Serendipity*, a very expressive word, which, as I have nothing better to tell you, I shall endeavor to explain to you: you will understand it better by the derivation than by the definition. I once read a silly fairy tale, called "The Princes of Serendip;" and as their Highnesses travelled, they were always making *discoveries, by accidents and sagacity, of things which they were not in quest of*: for instance, one of them discovered that a mule blind of the right eye had travelled the same road lately, because the grass was eaten only on the left side, where it was worse than on the right—now do you understand *Serendipity*?[3]

Kish commanded his son Saul to arise and go seek the lost asses. Saul went out to seek lost asses, but found a kingdom.

The outstanding example of Serendipity is the discovery of America. Columbus stumbled by accident and sagacity on an unknown continent. Although he made the voyage to the Indies four times, he died blissfully ignorant of a new world. No one now cares about Columbus' mistakes nor questions his motives

[1] Presidential address delivered before the Society of American Bacteriologists at its Thirty-sixth Annual Meeting, Chicago, December 28, 1934.

[2] Dated January 28, 1754.

[3] I first learned of Serendipity from my colleague, Dr. Walter B. Cannon.

which were for power and pelf. In his greed for gold and readiness for fight, he was the first of a long line of buccaneers who sailed the Spanish Main. He could not have made his glorious discovery of "something he was not in quest of" had he not ventured. He did, he dared, and triumphed despite opposition. Walpole tells us that there were three original Princes of Serendip, but he might have crowned Columbus King of the Realm.

Many a scientific adventurer sails the uncharted seas and sets his course for a certain objective, only to find unknown land and unsuspected ports in strange parts. To reach such harbors, he must ship and sail, do and dare; he must quest and question. These chance discoveries are called "accidental," but there is nothing fortuitous about them, for laggards drift by a haven that may be a heaven. They pass by ports of opportunity. Only the determined searcher, who is not afraid to seek, to work, to try, who is inquisitive and alert to find, will come back to his home port with discovery in his cargo

There have been those who would belittle the work of our patron saint Pasteur, by insinuating that as a Prince in Serendip, his results were achieved more by accident than by sagacity. What a distorted viewpoint of a genius whose unremitting toil and zeal gave so much to the happiness of the world and the security of life!

There is no agreement as to which of Pasteur's many achievements is the oustanding one. Some insist that his early work in crystallography was the most brilliant of his many contributions. It seems so to me. As a young student, he found that the right-handed crystals of tartaric acid polarized to the right, and the left-handed to the left; a mixture of the two neutralized each other. He was not satisfied simply to solve this problem of racemic acid which had long puzzled able scientists, but, as was his habit of mind, drew far reaching conclusions concerning the asymmetry of the molecule, etc. Others would place on top his work on spontaneous generation, on fermentation, on silk worm culture, on rabies, anthrax or anaerobic life. But though we may disagree as to the one greatest thing he did, there is unan-

imous agreement as to the outstanding thought he spoke, "Dans les champs de l'observation le hazard ne favorise que les esprits preparés."

There are many absurd stories about the discoveries that seem to come about through accidental blunders in the sense of Serendipity. It is said that Archimedes got the idea of specific gravity by noting the buoyancy of his body in water. How can anyone believe that Isaac Newton drew such deep deductions of the law of gravity and its universality by noting the fall of an apple as he lay musing on the grass in an orchard. Another one of these intriguing legends is told of James Watt, the teapot and the steam engine. Such things do not happen to Tom, Dick and Harry; yet Tom takes a bath, Dick sees an apple fall, and Harry observes the steam lift the cover of the teapot.

Kekulé, while dozing before the fire, saw the closed benzene ring. It is told how Descartes, lying in bed, evolved coördinate geometry. There are many authentic instances of poets, musicians and artists waking up out of a sound sleep with a glorious inspiration. Symphonies, pictures, gems of song, poems and great artistic creations have thus been inspired. But waking or sleeping, music is composed only by musicians, poetry by poets and art by artists; and creative art is given only to those rare souls whose genius works day and night, waking and sleeping. We find exactly the same situation in science where inspiration implies perspiration. The master word has always been and always will be "work." Chance and accidental discoveries in scientific research come only through preparation by toil and thought.

Every bacteriologist knows Gram's method and its fundamental importance. Few, however, know the story of its discovery. Gram, a Danish bacteriologist, working with Friedländer in Berlin, tells about it in the original publication of the method in Fortschritte der Medezin, March 15, 1884. He states in a foot-note that the method originated in the accidental observation that aniline-gentian violet preparations, after treatment with iodine, potassium iodide solution in alcohol, were quickly and fully decolorized; in his own words, "I was trying to find a double stain for kidney sections with blue nuclei and brown

cylindrical casts." Gram was seeking one thing and found another. The important thing is that he was adventuring; and he continued to seek, for he then tried the method on bacteria and found that some of them were Gram-positive and others Gram-negative.

One of the outstanding examples in our field of science is the discovery of vaccination. When Jenner was a young medical student at Sudbury, getting his education under his preceptor Ludlow, he heard a milkmaid say, "I cannot take smallpox because I have had cowpox." That belief was vague folklore at the time and had been noted and even used casually by others. We now know that it sensitized the mind of Jenner, who thought, worked and studied on that problem for a period of twenty-five years before he satisfied himself. He said, "I placed it upon a rock where I knew it would be immovable before I invited the public to take a look at it."

Pasteur took this fact that a mild and attenuated virus protects against a virulent disease and expanded it into a law. He said, "I have given to the word 'vaccination' an extension which I hope science will consecrate as an homage to the merit and immense services of Jenner." There was much of sagacity, and I can see little of accident in these discoveries.

One of the best examples of scientific Serendipity is the discovery, often told to me, by Emile Berliner of the principle of the microphone and telephone transmitter. He was chatting with a telegraph operator at a fire station in Washington on a hot, trying day. The telegraph key was clicking dots and dashes, when the operator casually remarked, "That's Jim on the other end. I can tell by the touch. A light pressure causes a different click from a firm pressure." Berliner, who had been working on electric circuits, went home to his garret laboratory and worked out the principle of loose contact.

Roentgen—at least so the story goes—was working with Crookes vacuum tubes and found the shadow picture of a key on a photographic negative hidden in his desk drawer. Instead of throwing it away as a spoiled plate, he studied how it came about, and now we have the miracle of the x-ray with all its usefulness.

You may think that the pioneer work on anaphylaxis started in the realm of Serendipity. If you will pardon a personal allusion, it was something like this. I was working on the standardization of diphtheria antitoxin and testing samples for potency and purity at the Hygienic Laboratory in Washington, where I was then Director. It was necessary to give subminimal lethal doses of toxin-antitoxin mixtures to determine the unit value. In consequence, we had many recovered guinea pigs on our hands. We soon found out that these recovered pigs died if used again to test the purity of diphtheria antitoxin. Theobald Smith knew this, and so did Park and probably others who were working in this field. I remember on one occasion asking my diener, Lindgren, to bring me some used pigs and he said, "Used pigs are no good for this test; they die."

Theobald Smith told this to Ehrlich who was on a visit to this country and upon his return to Frankfort he gave the problem to Otto to work out. Before this, Anderson, who was with me, told me of cases of sudden death in man following the injection of diphtheria antitoxin which was then hailed as a cure for asthma. That clicked. At first I though it was a toxone effect or some toxic substance. But step by step we eliminated toxone and other poisonous substances and came to the well known phenomenon of serum anaphylaxis. You may think we stumbled on this in the sense of Serendipity, but I am sure that if my good friend and colleague John F. Anderson were here, he would insist with me that the results were obtained through many years of work, and there was joy in that work.

Right here I should like to put in a word to beginners in research concerning the unexpected that is constantly cropping up in exploring the unknown. The natural tendency of the unprepared mind is to discard the unusual. It is dismissed because not wanted—it does not conform to the preconceived plan. The unusual and unexpected may be significant and perhaps have to wait for a blunderer from Serendip on a journey of adventure in quest of discovery. In other words, what may be called the byproducts in research are often more important than the primary aim of the study. The byproducts of industry are often its most

useful and profitable products; but we cannot have byproducts unless we make products.

The secondary effects of science itself may be even more valuable than the actual discoveries that are acclaimed. One of these secondary effects is the infiltration of the scientific method into the mode and thought and attitude of herd psychology. It has taught a frank facing of realities calmly and impersonally. It has shattered shackles and traditions and superstitions that have long chained our attitudes and methods of thought. It gives us a clearer understanding of the world in which we live and permits us to sound deeper into the well of truth. It is no longer impious to question, to doubt. All our problems, even those of an emotional nature, may now be discussed with calm detachment. This attitude has had a profound influence upon society, its morality, its ethics and its thinking. These adventures into the realm of reality are not accidental blunders in the sense of Serendipity, but inevitable byproducts of the scientific method.

The story of Ronald Ross is a striking example of sagacity and capacity, taking advantage of opportunity. He was poet, musician, mathematician and artist. Whatever he did, he did as a true and enthusiastic amateur. He tells us in his Memoirs that he studied medicine only to get a berth in the Indian Medical Service which would give him time to compose symphonies to excel those of Beethoven, write epics to rival Homer, and novels to replace Dickens. He invented a new system of mathematics, painted pictures, looked at sunsets; and made the rounds of the hospital as a chore. Then came the "accident." He took a vacation, returned to England and spent his time at the Army Medical School. He learned to use the microscope and the scientific method was revealed to him. From then on, he was inspired with research, although he still had time for the arts. He worked in the hot, humid and trying climate of Secunderabad on the problem of malaria, and in this isolation, while solving the puzzle of mosquito transmission, he composed his worthy epic "In Exile." He wrote to his wife, "I really think I have done the

mosquito theory at last. . . ." And then breaks out into song—a defiant rhapsody:

"This day relenting God
Hath placed within my hand
A wondrous thing; and God
Be praised. At His command,

Seeking His secret deeds
With tears and toiling breath,
I find thy cunning seeds,
O million-murdering Death.

I know this little thing
A myriad men will save.
O Death, where is thy sting?
Thy victory, O Grave?"

We recall how Beaumont took advantage of the accident which befell Alexis St. Martin. Schaudin objected to the order which required him to study the cause of syphilis, but his success came through his training and preparation for the task. Little did McCoy think when he discovered *Bacterium tularense* associated with a plague-like disease of rodents in California that he was disclosing the cause of tularemia and giving us the key to a new and important public health problem. Semmelweis, while attending the autopsy of a friend who had died of a septic infection, noted its similarity to the changes seen in childbed fever. This gave us the prevention of puerperal sepsis.

There are not only Princes in Serendip, but also Princesses. We have a Princess, a member of this Society. It would be difficult to conceive of two more diverse diseases than contagious abortion of cattle and malta fever of man; or two more different names than *Bacillus abortus* and the *Micrococcus melitensis*. Nevertheless, Alice Evans by simple technical processes brought them together.

Now we come to the army of modest retiring scholars who have never heard of Serendip, who have worked in monastic seclusion, actuated only by a desire to help mankind. These scientific workers, cloistered in their laboratories, are only too glad to

sacrifice themselves for the general good. One adds a small building block here, another aligns one there, still another puts in a stronger mortar. Together they are building a structure. The shape and uses of the whole cannot be foretold. It reminds me of the story of the three stone masons. When asked what they were doing, one said, "I am cutting stone;" another said, "I am making ten dollars a day;" and the third said, "I am building a cathedral." The hewers and drawers are not named at the laying of the corner stone nor at the dedication.

Scientists are sensitive flowers and many are born to blush unseen. It is to the modest, retiring, unknown and unknelled builders of our cathedral of science that William Mansfield Clark, the preceding President of this Society, dedicated his monograph on hydrogen ion concentration in these words: "To Fellow Workers in the Biological Sciences, Architects of Progress, Who Hew the Stone to Build Where Unseen Spires Shall Stand."

Bacteriologists need yield to none so far as the usefulness of their work and discoveries is concerned, for they have been of untold benefit to mankind. In no branch of human endeavor have greater benefits been bestowed. When the history of this epoch is written, the one outstanding accomplishment will be the conquest of disease, in which the members of this Society have played their parts. It has become a twice-told tale to recount the triumphs of preventive medicine. The control of disease has made life longer and surer. Longevity, however, is a poor index of progress. It is of no use to live longer unless we can live better. In other words, progress is measured in spiritual rather than material terms. Our next step, therefore, is mental and moral hygiene. Meanwhile, each of us adds his small piece to the mosaic of life, with the satisfaction that,

"When Earth's last picture is painted and the tubes are twisted and dried,
. . . only the Master shall praise us, and only the Master shall blame;
And no one shall work for money, and no one shall work for fame,
But each for the joy of working, and each, in his separate star,
Shall draw the Thing as he sees It for the God of Things as They are!"

A PROTRACTOR FOR COMPUTING THE GROWTH RATE OF BACTERIA

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When the logarithms of the number of cells in a growing culture of bacteria or yeasts are plotted against time, a straight line is obtained so long as the growth rate is constant. Ordinarily, we measure the growth rate by the generation time, i.e., by the time required for the average cell to double. It can be computed from the well-known equation, and for various successive time intervals, the different values may be averaged, or the smallest value, i.e., the fastest growth rate may be considered as the significant value. Either method is somewhat arbitrary, and it seems safer to average the various data of one experiment graphically, by plotting the logarithmic growth curve and drawing a straight line approaching as nearly as possible the experimental values at the point of most rapid growth. The slope of this curve is a measure of the growth rate.

We have designed a growth rate protractor which can be conveniently used for research or class work. The construction is very simple. One cell in 5 generations produces 32 cells, in 10 generations 1024 cells. If the generation time is 10 minutes, the logarithmic growth curve, starting at zero time with $\log 1 = 0$, is a straight line crossing $\log 32 = 1.505$ at $t = 50$ minutes, and $\log 1024 = 3.0103$ at $t = 100$ minutes. For all other generation times, the points where the 5 or 10 generations are completed can be easily calculated. Such a graph is shown in figure 1. It can be drawn on transparent celluloid, or cut in the form of a growth-rate protractor as indicated.

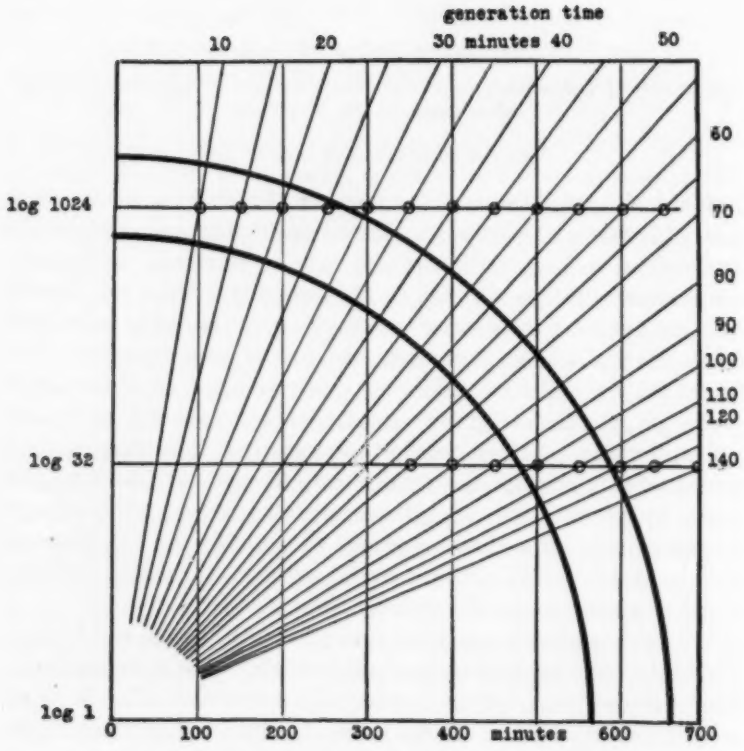

FIG. 1. CONSTRUCTION OF THE GROWTH RATE PROTRACTOR

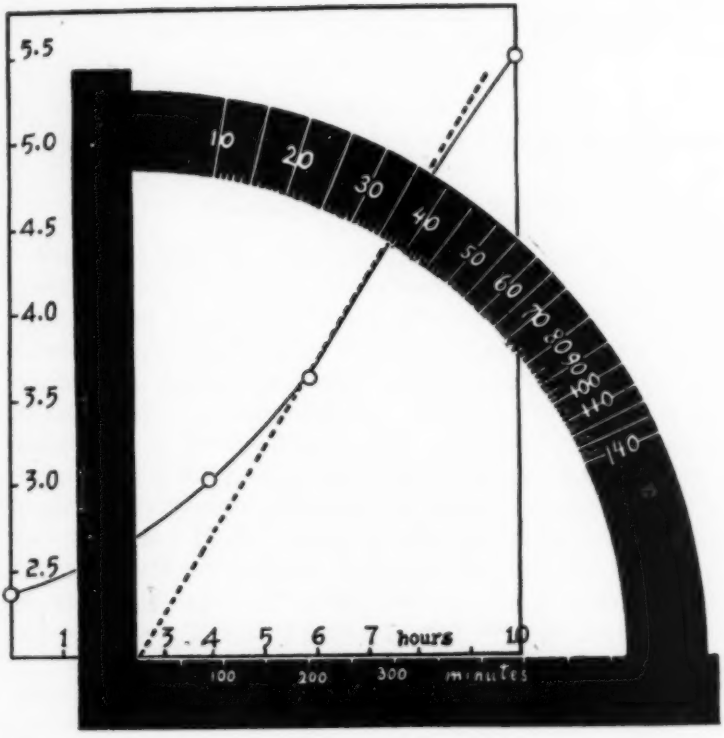

FIG. 2. APPLICATION OF THE GROWTH RATE PROTRACTOR TO A GROWTH CURVE OF *Pseudomonas fluorescens*

The dotted line is the extended curve of constant maximal growth rate

The application is shown in figure 2. The application requires that the scale be the same. However, it is easily seen that if the time scale is doubled, the true generation time is one-half of the direct reading of the protractor. The same is true with the ordinate. If any changes in scale must be made, it would be wise to use simple multiples.

A COMPARISON OF THE MAXIMAL GROWTH RATES OF VARIOUS BACTERIA UNDER OPTIMAL CONDITIONS

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When we speak in a general way of slowly growing or rapidly growing bacteria, we mean to indicate that under identical conditions, they grow at different rates. If we wish to compare widely different bacteria, it would not be wise, or even possible to choose identical conditions, because what is optimal for one species might be impossible for others. The only means for comparison is the maximal growth rate, the rate of cell division under optimal conditions. Here again, the question can be raised in each individual case whether the conditions are really the very best possible. It can never be absolutely denied that further improvements are possible, therefore this question need not be discussed at length. The compilation (table 1) gives the fastest growth rates which have been observed with the various species.

The growth rates are stated as generation times, i.e., as the average time required for the cells to double at the period of fastest growth.

Most of the data are from the literature; some have been computed from experiments by the author. Lack of space forbids quotation of the extensive literature; the references may be found in a thesis for the degree of MS. by the author, at Cornell University, with the same title as this paper.

The bacteria are grouped in genera arranged in the same order as in Bergey's Manual.

The compilation shows that the growth rate is frequently characteristic of the group, or genus. The representatives of the

TABLE 1

ORGANISM	MEDIUM	GENERATION TIME	AUTHORS
		minutes	
Azotobacter chroococcum	Sugar-mineral	240	Hunter, 1923
Azotobacter chroococcum	Sugar-mineral	348	Werkman, 1927
Azotobacter chroococcum	Sugar-urea	74	Burk and Lineweaver, 1930
Azotobacter chroococcum	Glucose broth	27–39	Mason, 1934
Rhizobium trifolii	Mannitol-mineral-yeast extract	174	Sherman, unpublished
Rhizobium trifolii	Same	101	Sherman, unpublished
Rhizobium meliloti	Same	107	Sherman, unpublished
Diplococcus mucosus	Milk	32	Heinemann and Glenn, 1908
Diplococcus pneumoniae I	Broth + serum	20.5	Chesney, 1916
Diplococcus pneumoniae I	Broth	24.5	Blake, 1917
Diplococcus pneumoniae I	Broth	28.7	Cullen and Chesney, 1918
Diplococcus pneumoniae I	Serum	29	Barber, 1919
Diplococcus pneumoniae I	Broth	31	Kligler, 1919
Diplococcus pneumoniae II	Broth	33	Chesney, 1916
Diplococcus pneumoniae II	Broth + serum	23	Blake, 1917
Diplococcus pneumoniae II	Broth	38	Cullen and Chesney, 1918
Diplococcus pneumoniae II	Glucose broth	30	Lord and Nye, 1919
Streptococcus lactis	Milk	23.5	Heinemann and Glenn, 1908
Streptococcus lactis	Milk	25	Foter, unpublished
Streptococcus lactis	Milk	26	Mason, 1934
Streptococcus lactis	Milk + peptone	37	Mason, 1934
Streptococcus lactis	Milk + glucose	26	Mason, 1934
Streptococcus lactis	Lactose broth	48	Rogers and Greenback, 1930
Streptococcus fecalis	Milk	23	Frazier and Whittier, 1931
Streptococcus fecalis	Glucose citrate broth	27	Foter, unpublished
Streptococcus fecalis	Milk	26.5	Mason, 1934
Streptococcus thermophilus	Milk	27	Frazier and Whittier, 1931
Streptococcus hemolyticus	Beef heart broth	32	Kligler, 1919

Streptococcus hemolyticus	Beef heart broth	32	Kligler, 1919
Streptococcus hemolyticus	Glucose broth	26	Foster, 1921
Streptococcus hemolyticus	Same + serum	34	Foster, 1921
Streptococcus liquefaciens	Milk	27	Mason, 1934
Streptococcus mastitidis	Milk + glucose	35–37	Mason, 1934
Staphylococcus aureus	Broth	27	Graham-Smith, 1920
Staphylococcus aureus	Broth	30	Barnes, unpublished
Staphylococcus aureus	Glucose broth	32	Mason, 1934
Staphylococcus albus	Glucose broth	24–25	Mason, 1934
Vibrio cholerae	Broth	21.2	Buchner, Longard and Riedlin, 1887
Vibrio cholerae	Broth	38	Henrici, 1928
Vibrio costatus	Broth	42	Lloyd and Cranston, 1930
Serratia marcescens	Milk	37	Heinemann and Glenn, 1908
Pseudomonas fluorescens	Broth	40	Müller, 1903
Pseudomonas fluorescens	Broth	40	Rahn, 1906
Pseudomonas fluorescens	Glucose broth	34–34.5	Mason, 1934
Pseudomonas pyocyanea	Broth	34	Mason, 1934
Pseudomonas pyocyanea	Lactose broth	34	Mason, 1934
Pseudomonas pyocyanea	Glucose broth	31	Mason, 1934
Erwinia carotovora	Broth	57	Mason, 1934
Erwinia carotovora	Glucose broth	42	Mason, 1934
Phytomonas angulata	Broth, 25°	76	Wolf and Foster, 1921
Phytomonas tabacum	Same	81	Wolf and Foster, 1921
Phytomonas sojae	Same	82	Wolf and Foster, 1921
Phytomonas glycinea	Same	95	Wolf and Foster, 1921
Phytomonas campestris	Same	165	Wolf and Foster, 1921
Phytomonas campsetre	Same	74	Wernham, unpublished
Phytomonas campsetre	Same	98	Mason, 1934 } see text
Phytomonas campsetre	Glucose broth	78	Mason, 1934 } see text

TABLE 1—*Concluded*

ORGANISM	MEDIUM	GENERATION TIME	AUTHORS
		minutes	
Phytomonas phaseoli	Broth	150	Mason, 1934
Phytomonas phaseoli	Bean broth	160	Mason, 1934 } see text
Phytomonas phaseoli	Glucose broth	138	Mason, 1934
Lactobacillus acidophilus	Milk	67–87	England, 1932
Lactobacillus acidophilus	Milk	66	Foter, unpublished
Lactobacillus bulgaricus	Milk	39	Frazier and Whittier, 1931
Lactobacillus bulgaricus	Yeast extract	37	Cohen and Clark, 1919
Lactobacillus bulgaricus	Milk	74	Mason, 1934
Lactobacillus bulgaricus	Milk + glucose	41–75	Mason, 1934
Lactobacillus bulgaricus	Same + peptone	50	Mason, 1934
Lactobacillus bulgaricus	Same + tomato juice	38–40	Mason, 1934
Lactobacillus casei	Milk	38	Frazier and Whittier, 1931
Lactobacillus Delbrücki	Wort, 45°	83	Slator, 1916
Lactobacillus pentoaceticus	Yeast extract	67	Anderson, Fred and Peterson, 1920
Pasteurella lepiseptica	Broth + blood	24.4	Webster, 1925
Escherichia coli	Broth	17	Hüne, 1908
Escherichia coli	Broth	17	Barber, 1908
Escherichia coli	Broth	16.5	Sherman and Holm, 1922
Escherichia coli	Broth	17	Curran, 1925
Escherichia coli	Milk	12.5	Frazier and Whittier, 1931
Escherichia coli	Lactose broth	16	Saito, 1907
Escherichia coli	Lactose + peptone	16	Winslow, Walker, and Sutermeister, 1932
Escherichia communior	Broth	16	Hehewerth, 1901
Aerobacter aerogenes	Broth	18	Curran, 1925
Aerobacter aerogenes	Milk	18	Conn and Esten, 1904
Aerobacter aerogenes	Peptone	22	Cohen and Clark, 1919

Aerobacter aerogenes	Peptone	22	Cohen and Clark, 1919
Aerobacter aerogenes	Peptone	30	Mason, 1934
Aerobacter aerogenes	Peptone + glucose	17.2–17.4	Mason, 1934
Proteus vulgaris	Broth	21.5	Stark, 1927
Proteus vulgaris	Peptone-phosphate	40	Cohen and Clark, 1919
Salmonella enteritidis	Broth, 42°	21.5	Lane-Claypon, 1909
Salmonella paratyphi	Broth	23	Reichenbach, 1911
Salmonella paratyphi	Peptone	28	Gordon, 1934
Salmonella suipestifer	Broth	26	Wilson, 1922
Eberthella typhosa	Broth	23.5	Müller, 1895
Eberthella typhosa	Bile + pus	24.5	Pies, 1907
Eberthella typhosa	Peptone + glucose	33	Penfold and Norris, 1912
Eberthella typhosa	Glucose broth	29	Coulter and Isaacs, 1929
Shigella dysenteriae	Peptone-phosphate	37	Cohen and Clark, 1919
Shigella dysenteriae	Milk	23	Heinemann and Glenn, 1908
Bacillus mycoides	Broth	28	Ward and Frankland, 1895
Bacillus megatherium	Broth	31	Henrici, 1928
Bacillus cereus	Broth	18.8	Stark, 1927
Bacillus cereus	Glucose broth	17–24.5	Mason, 1934
Bacillus thermophilis	Broth, 55°	18.3	Wallace, 1924
Bacillus thermophilis	Glucose broth, 55°	16	Hansen, 1932
Bacillus subtilis	Glucose broth	26–32	Mason, 1934
Clostridium botulinum	Glucose broth	35	Wagner, Mayer and Dozier, 1925
Clostridium Welchii	Milk	35	Frazier and Whittier, 1931
Clostridium amylobacter	Corn mash	51	McCoy, Fred, Peterson and Hastings, 1926
Corynebacterium diphtheriae	Serum + glucose broth	34	Mason, 1934
Corynebacterium pseudodiphtheriae	Broth	37	Henrici, 1928

colon-aerogenes group are the most rapidly growing bacteria, doubling in less than twenty minutes. Some of the larger spore-forming bacilli also reach this rate. Eberthella, Salmonella and Proteus multiply not quite so readily, requiring twenty to thirty minutes. This is also the rate of *Vibrio cholerae*; the other Vibrios mentioned may not have had optimal conditions.

The staphylococci and streptococci have a generation time of twenty-five to thirty-five minutes as a rule. The same holds true for the diplococci, with one exception. Much slower are the polar flagellates. *Pseudomonas fluorescens or Ps. pyocyanea* could not be made to double in less than thirty minutes, and forty minutes is more nearly the average. Slower still is the growth of the plant pathogenic group. In all experiments by the author with *Phytomonas phaseoli* and *Ph. campestris*, the growth curve consisted of two sigmoid curves, one following the other. Since this was observed in 5 experiments with this genus, and never with any other genus, it must be considered characteristic of Phytomonas. This makes a precise statement of the generation time impossible; but it is more than one hour.

The large sporeformers, with the exception of *B. megatherium*, multiply very rapidly, the fastest of all being the thermophilic species. Mudge considered even a generation time of about ten minutes as possible, but Hansen's extensive plate counts showed sixteen minutes to be the limit of his species.

Corynebacterium is a fairly slowly growing bacterium; the related Mycobacteria are much slower, the pathogenic species even more so than the parasitic organisms. The slowest of all is probably the group of Nitrobacter and Nitrosococcus, the true prototrophic bacteria.

Azotobacter grows more rapidly in media containing organic nitrogen than in a medium lacking this constituent. Not enough data were available to determine whether or not this is also true of Rhizobium.

SUMMARY

Under the most favorable conditions, different bacterial species show decided differences in growth rate.

In many cases, the growth rate is characteristic of the genus, and it seems permissible to speak of a genus as being rapidly growing, slowly growing, or of a medium growth rate. The genus Bacillus contains species with rapid as well as with medium growth rates.

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THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI IN RELATION TO THE SOURCE OF STRAINS AND TO CULTURAL REACTIONS

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The dissolving action, which beta hemolytic streptococci exert on solid human fibrin, is dependent upon an extracellular substance freely excreted by the living, growing bacterial cells (Tillett and Garner, 1933). Investigations of the lytic phenomenon have revealed certain special characteristics which pertain to the occurrence and nature of the reaction and to the specific immunological response of patients (Tillett, Edwards and Garner, 1934; Van Deventer and Reich, 1934; Hadfield, Magee and Perry, 1934; Dennis and Berberian, 1934; Madison, 1934; Garner and Tillett, 1934).

Recent biochemical studies (Garner and Tillett, 1934) indicate some of the properties of the streptococcal fibrinolysin, which, although enzymatic in its action, is not analogous to proteolytic ferments such as trypsin. For example, one difference between the streptococcal product and trypsin lies in the fact that the fibrinolysin, with the experimental procedures employed, produces no detectable changes in either casein, gelatin, or peptone. Furthermore, even when fibrin is used for the substrate, the special selectivity of the fibrinolysin is exemplified by the fact that the clot of normal human blood is highly susceptible, while the coagulum from the blood of small laboratory animals is not visibly affected. Van Deventer and Reich (1934) have reported the results of tests performed with the fibrin from several different animal species and found that only the blood of rhesus monkeys approximates that of man in susceptibility.

In addition to specificity with regard to substrate, the type of bacterium, which elaborates fibrinolysin, seems to be sharply limited. With the possible exception of the slow, irregular, liquefying effect of some strains of *Staphylococcus aureus*, fibrinolysis is a unique function of streptococci, and not demonstrable with other species of bacteria ordinarily associated with infection in man. A study has not been made of the many types of microorganisms occurring in animals or nature.

Concerning streptococci, only strains producing the *beta type of hemolysis*, as defined by Brown (1919), have been found to be active. Subsequent reference will be made to the importance of determining the type of hemolysis.

One other element of specificity, which will be elaborated in this paper, is brought out by the fact that, whereas beta hemolytic streptococci isolated from patients are usually highly fibrinolytic, strains of animal origin are often inert in this respect.

In pursuing studies of the fibrinolytic phenomenon, two hundred and seventeen strains have been acquired for bacteriological study. The strains were selected with particular attention to the source, and each one was of the beta hemolytic type. With a few exceptions, the human strains were isolated in this laboratory from patients in the wards of the hospital. The animal strains were given to us by Dr. J. Howard Brown of the Johns Hopkins Medical School, by Dr. Philip R. Edwards of the Kentucky Agricultural Experiment Station, and by Dr. R. C. Lancefield of the Hospital of the Rockefeller Institute. These investigators have also permitted us the use of results contained in published reports and in personal communications, thus insuring correct identification of the strains. It is a pleasure to acknowledge indebtedness to Dr. Brown, Dr. Edwards, and Dr. Lancefield for their generous assistance and suggestions.

The purpose of the investigation has been to correlate the fibrinolytic activity of the strains with other biological and biochemical properties of streptococci. The strains have been evaluated with respect to human pathogenicity on the basis of the presence or absence of fibrinolytic capacity in relation to the origin of the strain. In addition, the lytic potency of the several

active cultures has been determined with consideration of the type and severity of the infection produced in patients. The limitations, which restrict the interpretation of the results, will be referred to as the observations are described.

The investigation has been further extended to include, where applicable, a comparison of fibrinolytic activity with selected biochemical cultural characteristics which are commonly utilized in the bacteriological classification of hemolytic streptococci.

Although the serological classification, devised by Lancefield (1933), has not been carried out in this laboratory, Dr. Lancefield has very kindly permitted the incorporation of her published results in the tables of this report. Since many of the animal strains used by us were sent by Dr. Lancefield, the characterization of the strains is made more complete by tabulating all of the results together.

One of the striking features of the fibrinolytic phenomenon is the rapidity with which the fibrin may be dissolved. Under optimal experimental conditions, liquefaction occurs in a few minutes. However, the time necessary to effect complete lysis, is not the same with all strains (Tillett and Garner, 1933; Hadfield, Magee and Perry, 1934; Madison, 1934). The rate of dissolution is, therefore, an approximate quantitative measure of the amount of fibrinolysin produced by the test culture. For practical purposes, the lytic potency of cultures has been graded as follows:

++++ indicates complete lysis in less than 30 minutes
+++ indicates complete lysis in 30 to 60 minutes
++ indicates complete lysis in 1 to 3 hours
+ indicates complete lysis in 3 to 24 hours
− indicates no lysis in 24 hours

MATERIALS AND METHODS

The details of the method of demonstrating the fibrinolytic phenomenon have been described elsewhere. Briefly stated, it is as follows: 0.2 cc. of oxalated human plasma (0.02 gram potassium oxalate per 10 cc. of blood) + 0.8 cc. of physiological salt solution + 0.5 cc. of a sixteen- to twenty-hour broth culture

of the test organism + 0.25 cc. of 0.25 per cent $CaCl_2$ solution. The tubes are well shaken after the introduction of each additional constituent, and are placed in the water bath at 37.5°C. after the addition of $CaCl_2$. With potent cultures and susceptible plasma, coagulation occurs in six to fifteen minutes and complete dissolution in about six to ten minutes.

In testing many of the doubtful or negative strains, a fibrin clot composed of fibrinogen, chemically isolated from blood, coagulated with solutions of thrombin, also derived from blood, has been employed. The reason for this is based on the fact that the fibrinogen-thrombin coagulum is more susceptible to lysis than the clot of whole plasma. The method of obtaining fibrinogen and thrombin solutions has been previously described (Tillett and Garner, 1933).

Cultures. Plain, unbuffered, meat infusion broth, pH 7.6, containing 0.05 per cent glucose, has been the culture medium of choice. The cultures were allowed to incubate for sixteen to twenty-four hours and used promptly.

The strains, derived from patients, originally came from colonies picked from the surface or depths of blood agar cultures. The fibrinolytic test was, in the greatest number of instances, performed with the first broth subculture.

Strains received from other laboratories have been, in many instances, in artificial cultivation for several years. The data concerning these strains will be appropriately recorded.

Biochemical reactions. Sugar fermentations. The culture base consisted of plain meat infusion broth devoid of the 0.05 per cent glucose. Solutions of lactose, mannitol, salicin, sorbitol, and trehalose (0.5 per cent) were made in the broth. Trials with other media recommended for fermentative tests resulted in such great variations in the abundance of growth that the reactions were unsatisfactory. That the muscle sugar, which may be present in meat broth, was negligible, was indicated by appropriate controls.

After inoculation, the cultures were allowed to incubate for 5 days. Methyl red indicator was added after incubation.

pH in 1 per cent glucose. Glucose was added to meat infusion broth (pH 7.6). Fifty-cubic-centimeter centrifuge tubes, containing 25 cc. of medium, were inoculated and incubated for three days. The tubes were then centrifuged at high speed and the clear supernatant fluid was transferred to appropriate test tubes. Brom-cresyl-green indicator was employed. The standards, continually stored in the dark, were prepared within a month of use.

Hydrolysis of sodium hippurate. Cultures were allowed to grow for forty-eight hours. The usual procedure was employed.

SOME NOTES ON TECHNICAL PROCEDURES WHICH INFLUENCE FIBRINOLYSIS

Before proceeding with a description of the observations with which this report is primarily concerned, it is desirable to record briefly certain conditions which influence the fibrinolytic activity of cultures. Some of these factors are being given special study and the results will be presented in subsequent publications; others have not yet been investigated.

Identification of strains. As previously stated, fibrinolysis appears to be limited to hemolytic streptococci of the beta type. Frequently hemolytic colonies have been picked from the surface of solid media and found to be non-fibrinolytic. When these negative cultures were subcultured in poured blood agar, properly incubated, and finally chilled in the icebox for twenty-four hours, it was often found that they belonged to types classified by Brown as alpha or alpha prime (Brown, 1919), or as double-zoned (Brown, 1934). It is evident, therefore, from this experience that, when a strain of streptococcus proves incapable of liquefying fibrin, the type of hemolysis should be correctly established.

Culture medium. The rôle of environmental factors and culture media, in increasing or reducing the amount of fibrinolysin produced by streptococci, is being given special study. Although the results are incomplete, it is of interest to note, as one example, the effect which defibrinated rabbit's blood may have

upon a strain. Two drops of sterile rabbit's blood was added to 10 cc. of plain broth. Scarlet fever strain J was used:

	Dissolution time
1. First culture in plain broth	45 minutes
2. First culture in blood broth	15 minutes
3. Second culture plain broth (Transfer of No. 1)	1 hour 15 minutes
4. Second culture blood broth (Transfer of No. 2)	12 minutes
5. Third culture plain broth (Transfer of No. 3)	1 hour 40 minutes
6. Third culture blood broth (Transfer of No. 4)	16 minutes
7. Transfer of No. 5 to blood broth	50 minutes
8. Transfer of No. 7 to blood broth	30 minutes

The transfers were made at weekly intervals.

From the results it may be seen that the subcultures in plain broth in sequence tended to weaken in activity whereas those kept in blood broth maintained maximum potency. These same conditions do not, however, hold for all strains. With some, maximum activity is continually retained regardless of the type of culture media used. With other weakly lytic strains, no enhancement of activity has yet been accomplished. The lability in the function of some strains and the stability of others has been frequently observed.

Relation of lytic potency to duration of cultivation outside the body. It may be briefly stated that some strains have been found to be highly lytic when first obtained from a patient while after several weeks or months of repeated subculturing, the dissolution time is markedly slowed. The human strains used in this investigation were, with few exceptions, tested on the first or second subculture after isolation. This procedure was an attempt to approximate by the laboratory test the potential lytic capacity of the strain when causing infection.

Importance of time factor in preparing a test. This factor in the technical procedure refers to the effect on dissolution rate of the length of time plasma culture mixtures are allowed to remain liquid before the addition of $CaCl_2$. The following protocol illustrates the nature of the results.

	TIME OF INCUBATION BEFORE ADDITION OF $CaCl_2$	DISSOLUTION TIME	
		Strain Co	Strain St.
1	Immediate	7 minutes	50 minutes
2	15 minutes	6 minutes	1 hour
3	30 minutes	20 minutes	1 hour 50 minutes
4	45 minutes	38 minutes	5 hours
5	60 minutes	1 hour 30 minutes	Negative
6	2 hours	Negative	Negative

These results indicate that even in normal plasma there is a small amount of antifibrinolytic substance which, during incubation, gradually "neutralizes" the lytic principle. The inhibiting factor in normal blood was also indicated by previous experiments (Tillett and Garner, 1933) which demonstrated that the fibrin formed by clotting fibrinogen solutions with partially purified thrombin was dissolved more rapidly than the coagulum of whole plasma clotted with $CaCl_2$.

In view of the results just described, it has been the rule, in this laboratory, to prepare only ten or twelve tests at a time, thus minimizing the effect of the normal antifibrinolysin.

Selection of plasma. Oxalated plasma should be employed within forty-eight hours after removal from the body. In older samples coagulation with $CaCl_2$ is inhibited or very much delayed.

It is also important to use only plasma, the fibrin clot of which is known to be highly susceptible to lysis. Previous reports (Tillett, Edwards and Garner, 1934) have demonstrated that the blood of individuals convalescent from acute streptococcus infections are often highly resistant to fibrinolysis and that about 25 per cent of normal persons possess varying degrees of the same resistance. In view of this fact it is obviously possible to evaluate the lytic potency incorrectly by testing with resistant plasma.

LYTIC ACTIVITY OF STRAINS ISOLATED FROM PATIENTS

The results, which are summarized in table 1, were obtained with cultures isolated in this laboratory. The cultures were

promptly identified and fibrinolysis was determined with the first broth transplant. Since the individuals, from whom the streptococci were obtained, were patients in the hospital, it was possible to establish with considerable certainty the relationship of the organisms to the type of infection. The fulfillment of these conditions has been considered helpful in evaluating results which have, at best, the potential fallacies inherent in

TABLE 1

Summary of fibrinolytic test with human strains of different disease sources

SOURCE OF CULTURE	NUMBER OF CULTURES	++++ DISSOLUTION IN LESS THAN 30 MINUTES		+++ DISSOLUTION IN 30 MINUTES TO 1 HOUR		++ DISSOLUTION IN 1 HOUR TO 3 HOURS		+ DISSOLUTION IN LONGER THAN 3 HOURS		− NEGATIVE, 24 HOURS	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Septicemia	17	13	76.3	2	11.3	2	11.3	0	0	0	0
Acute suppurative diseases*	21	16	76.1	2	9.5	3	14.2	0	0	0	0
Erysipelas	9	5	55.5	1	11.1	3	33.3	0	0	0	0
Acute tonsillitis	22	9	40.9	8	36.3	5	22.7	0	0	0	0
Scarlet fever	31	10	32.2	10	32.2	9	29.0	1	3.2	1	3.2
Acute nephritis	8	8	100.0	0	0	0	0	0	0	0	0
Acute rheumatic fever	7	3	42.8	3	42.8	1	14.2	0	0	0	0
Chronic conditions, routine cultures and normals	25	6	24.0	4	16.0	10	40.0	5	20.0	0	0
Total	140	70	50.0	30	21.4	33	23.5	6	4.2	1	0.7

* This group consists of acute cases of meningitis, peritonitis, empyema, osteomyelitis, mastoiditis, and deep seated abscesses.

interpreting any test performed *in vitro* in terms of activity occurring *in vivo*.

The results presented in the table indicate that active fibrinolysin is characteristically elaborated by strains of beta hemolytic streptococci which are associated with human disease; one hundred and thirty-nine out of one hundred and forty strains were fibrinolytic. The one negative strain came from the throat culture of a case of scarlet fever. A single colony was picked from blood agar. In other cultural respects this strain

conforms to the human variety; it produces beta type of hemolysis, it belongs to the pyogenes group of Holman (1916), it ferments trehalose and not sorbitol (Edwards, 1932, 1933), it belongs to Group A in the serological classification of Lancefield (1933); toxin production has not yet been estimated.

With respect to the potency of the cultures, the number of strains representing each type of infection is too few to justify statistical analysis. It is interesting to note, however, that among the strains, which were derived from conditions particularly characterized by bacterial invasion (septicaemia and acute suppurative processes), approximately 87 per cent belong to the more highly active groups (++++ and +++), whereas only 13 per cent are weakly lytic (++ and +). In contrast, the low grade disorders and normal carriers supplied strains, the majority of which (60 per cent) belong to the weakly lytic groups (++ and +) and 40 per cent to the more rapidly acting groups (++++ and +++).

Although all of the strains included in table 1 have not been tested for fermentative action on sugars, several examples of each group have been so used. Lactose and salicin were regularly fermented, but mannitol was unaffected. This selectivity is commonly encountered with human strains of hemolytic streptococci and is characteristic of strains designated *S. pyogenes* (Holman, 1916). Furthermore, each of these strains fermented trehalose but failed to attack sorbitol (Edwards, 1932, 1933). In order to determine whether or not there is an association between fibrinolytic activity and the sugar fermentation reactions used by Holman (1916) in the classification of streptococci, a few strains have been available, which, because of their special reactions, are separated from the pyogenes group. These cultures consist of two strains of *Str. alactosus*, three strains of *Str. infrequens*, four strains described as similar to *Str. equi*, and one strain of *Str. anginosus*. The organisms have been in artificial cultivation for periods of time varying from a few weeks to several years; all but two (Y. and Scott) were obtained at autopsy. Because of the interest in, and probable importance of, the source of the strain in relation to biological characteristics,

the origin of each culture is given in table 2 along with the results of biochemical reactions.

The results indicate that fibrinolysis is not necessarily associated with any particular pattern of fermentative action but does appear to be related to the disease-producing capacity of the strain when isolated. For example *Str. alactosus* strain S. H. caused acute peritonitis in the patient and is maximally fibrinolytic. *Str. alactosus* strain Y., on the other hand, had no demonstrable relationship with the endocarditis (*Streptococcus viridans*) from which the patient suffered. Similar circumstances hold for the four *Str. infrequens* strains. The negative strain, 13571–I, in this group was one of five species of bacteria obtained at autopsy performed thirty-four hours after death of the patient. The findings undoubtedly represent post-mortem invasion, since the patient's clinical condition was not that of infection.

Since the typical *Str. equi* fails to attack either sorbitol or trehalose, the three strains in table 2, because of their atypical action in these sugars, are designated as equi-like. The relation of the strains to disease is not entirely clear. The hemolytic streptococci were probably part of a mixed infection.

A third group of human strains, which are considered separately, consists of seven cultures of so-called *Streptococcus epidemicus*. The strains, received from Dr. Philip Edwards, were derived from patients suffering from septic sore throat, which occurred in milk-borne epidemics. The results of biochemical reactions and fibrinolytic tests are presented in table 3.

The high degree of fibrinolytic potency of this group of epidemic strains is striking. It is also interesting to note that the organisms have been in artificial laboratory cultivation for several years.

The observations, which have just been described, were made with one hundred and fifty-seven strains of beta hemolytic streptococci derived from human sources. The experiments were carried out with as strict adherence as possible to conditions which were outlined in the earlier part of this article and are deemed important. One hundred and fifty-four of the strains possessed fibrinolytic activity. Of the three negative cultures,

TABLE 2

Fibrinolytic activity and biochemical reactions with specially classified human strains

CLASSIFICATION OF STRAIN OF STREPTOCOCCI	STRAIN	SOURCE	DISEASE	FERMENTATION OF			pH in 1 per cent glucose	HYDROLYSIS OF SODIUM HIPPURATE	FERMENTATION OF		FIBRINOLYTIC TEST
				Lactose	Mannitol	Salicin			Sorbitol	Trehalose	
Alactosus	S.H.	Peritoneal	Peritonitis exudate	−	+	+	4.8	−	−	+	++++
	Y.	Extracted tooth	Endocarditis streptococcus viridans	−	+	+	4.7* 4.4†	−	+	+	Negative
Infrequens	8596	Blood. Autopsy	Meningitis	+	+	+	4.9	−	−	+	++++
	8606	Blood. Autopsy	Pneumonia, septicemia	+	+	+	4.9	−	−	+	++++
	Scott	Tonsil	Acute nephritis	+	+	+	4.9	−	−	+	++++
	13571–I	Blood. Autopsy	Renal rickets	+	+	+	4.6	−	+	+	Negative
Equi-like	12436	Blood. Autopsy	Congenital syphilis	−	−	+	4.5		−	+	+
	13384	Lung. Autopsy	Pneumonia type II	−	−	+	5.4	−	−	+	++
	13480	Bronchus. Autopsy	Pertussis	−	−	+	5.6	−	−	+	++
Anginosus	8003	Blood. Autopsy	Puerperal sepsis	+	−	−	5.1	−	−	+	++++

* Result obtained by Dr. J. Howard Brown.

† Result obtained in this laboratory.

one was from a case of scarlet fever; in the other two instances, the organisms were not obtained from the sites of active infection and were, in all probability, not associated with illness in the patients.

Hadfield, Magee, and Perry (1934) tested twenty-nine human strains and divided them, according to fibrinolytic potency into three groups, the least active of which caused lysis slowly and incompletely in twenty-four hours. Eighteen of the strains belonged to the weakly active group. The more potent strains were from cases with severe infection. Recently Madison (1934)

TABLE 3

Fibrinolytic Activity and Biochemical Reactions with Human Strains Derived from Patients with Septic Sore Throat (Streptococcus Epidemicus)

STRAIN	LABORATORY	EPIDEMIC	FERMENTATION OF			pH IN 1 PER CENT GLUCOSE	HYDROLYSIS OF SODIUM HIPPURATE	FERMENTATION OF		FIBRINOLYTIC TEST
			Lactose	Mannitol	Salicin			Sorbitol	Trehalose	
E_{22}	Edwards	Baraboo, Wis.	+	−	+	4.9	−	−	+	++++
E_{23}	Edwards	Westerlo, N. Y.	+	−	+	5.0	−	−	+	++++
E_{24}	Edwards	Lee, Mass.	+	−	+	4.8	−	−	+	++
E_{59}	Edwards	Kirkland Lake, Ontario	+	−	+	5.0		−	+	++++
E_{60}	Edwards	Kirkland Lake, Ontario	+	−	+	5.1		−	+	++++
E_{64}	Edwards	Kirkland Lake, Ontario	+	−	+	4.9		−	+	++++
E_{65}	Edwards	Kirkland Lake, Ontario	+	−	+	4.9		−	+	++++

reported results obtained with one hundred and ninety-three strains of hemolytic streptococci, one hundred and fifty-five of which were designated as being from human sources. Madison further subdivided the human strains into those—32—from internal human tissues of which 94 per cent were lytic, and those —123—from superficial human tissues of which 17 per cent were lytic. Although the authors just cited found a much higher percentage of weakly active or negative strains among the cultures of human origin than have been encountered in this laboratory, both articles comment upon the high potency of streptococci obtained from severe infections.

LYTIC ACTIVITY OF ANIMAL STRAINS

The biological, biochemical, and serological characteristics, which distinguish animal strains of hemolytic streptococci, have been extensively investigated by others and reviews of the literature may be found in numerous articles (Lancefield, 1933; Edwards, 1932, 1933; Brown, Frost and Shaw, 1926). Final identification rests upon the results of several different biological and biochemical procedures, no one of which alone is adequate. The extensive studies of Lancefield (1933) on serological classification based on reactions of the somatic fraction C with immune rabbit sera offers a single test which separates human from animal strains and further subdivides the animal group. The observations of Lancefield have been recently confirmed by Edwards (1934).

In a previous report (Tillett and Garner, 1933) it was shown that only 3 out of 18 animal strains of beta hemolytic streptococcus were capable of dissolving human fibrin clot. Since the original report, additional strains have been obtained and studied in detail. The results given in table 4 were obtained with animal strains, exclusive of the bovine group.

From the table it may be seen that only two of the strains are capable of dissolving human fibrin clot. One of these (K158C) conforms in both biochemical reactions and in serological classification to a human strain. That fibrinolysis occurs, is, therefore, to be expected. The other active animal strain (K61) behaves in every way, except in the fibrinolytic test, as an animal strain and seems to be, therefore, an exception.

In view of the fact that a preponderant majority of human strains of hemolytic streptococci were found to possess the lytic function whereas animal strains were usually devoid of this activity, special attention has been given to strains derived from cows or milk. It is unnecessary to discuss the practical importance which is associated with these veterinary strains. Hemolytic streptococci of dairy origin present the problem of whether they may be pathogenic for men and cause epidemics of septic sore throat, or are probably harmless for man. The investiga-

tions of others, which need not be reviewed here, indicate that, by the determination of several biochemical reactions, the pathogenic significance of the organisms may be evaluated. If the strains are low-acid-producing, and incapable of hydrolyzing sodium hippurate, the tests for fermentation in trehalose and

TABLE 4

Fibrinolytic activity and biochemical reactions with animal strains (exclusive of bovine group)

STRAIN	LABORATORY	ANIMAL SOURCE	FERMENTATION OF			pH IN 1 PER CENT GLUCOSE	HYDROLYSIS OF SODIUM HIPPURATE	FERMENTATION OF		LANCEFIELD SEROLOGICAL CLASSIFICATION	FIBRINOLYTIC TEST
			Lactose	Mannitol	Salicin			Sorbitol	Trehalose		
R_{226}	Lancefield	Rabbit	+	−	+	4.7	−	+	−	Group C	−
K_{158A}	Lancefield	Rabbit	−	−	+	4.4	+	−	+	Group B	−
K_{158B}	Lancefield	Rabbit	+	−	+	4.8	−	+	−	Group C	−
K_{158C}	Lancefield	Rabbit	+	−	+	5.1	−	−	+	Group A	++++
K_{158D}	Lancefield	Rabbit	+	−	+	4.8	−	+	−	Group C	−
K_{158E}	Lancefield	Rabbit	+	−	+	4.8	−	+	−	Group C	−
K_{158F}	Lancefield	Rabbit	+	−	+	4.7	−	+	−	Group C	−
Br_1	Brown	Rabbit									−
K_{56}	Lancefield	Guinea pig	+	−	+	4.7	−	+	−	Group C	−
K_{61}	Lancefield	Guinea pig	+	−	+	4.8	−	+	−	Group C	+++
K_{64}	Lancefield	Guinea pig	+	−	+	4.8	−	+	−	Group C	−
K_{104}	Lancefield	Guinea pig	+	−	+	4.8	−	−	−	Group C	−
P_{454}	Lancefield	Guinea pig	+	−	+	4.8	−	+	−	Group C	−
J_{20}	Lancefield	Guinea pig	+	−	+	4.8		+	−		−
Br_2	Brown	Guinea pig									−
H_1	Own	Horse	+	−	+	4.6					−
H_2	Own	Horse	+	−	+	4.5		+	−		−
Reid	Brown	Turkey dressing	+	+	+	4.3		+	+		−

sorbitol, as devised by Edwards (1932, 1933), are of great value. The serological studies of Lancefield indicate that, with immune sera of the different groups, cultures may be definitely classified. With the results of others in evaluating the bovine strains available, studies on fibrinolysis have been made and the findings tabulated in connection with the identifying reactions.

Forty-three strains from either milk or cows have been investigated and divided into two groups: those belonging to the so-called epidemicus groups, and those considered harmless for man.

TABLE 5

Fibrinolytic activity and biochemical reactions with cultures from cows or milk, classified as human pathogenic strains

STRAIN	LABORATORY	EPIDEMIC	SOURCE	FERMENTATION OF			pH IN 1 PER CENT GLUCOSE	HYDROLYSIS OF SODIUM HIPPURATE	FERMENTATION OF		FIBRINOLYTIC TEST
				Lactose	Mannitol	Salicin			Sorbitol	Trehalose	
Lee 27A	Brown	Lee, Mass., 1928	Cow's udder	+	−	+	5.0	−	−	+	++++
C 108	Brown	Boston, Mass., 1917	Cow's udder	+	−	+	5.0	−	−	+	++++
C 2b	Brown	Canton, Mass., 1913	Milk	+	−	+	5.0	−	−	+	++++
1452	Brown	1927	Cow's udder	+	−	+	5.0	−	−	+	+++
W.Mkt.I	Brown	No epidemic	Milk	+	−	+	5.4* 4.9†	−	−	+	++++
V 10	Lancefield		Cow's udder	+	−	+	5.1	−	−	+	++++
E 56	Edwards	Kirkland, Lake Ont.	Cow's udder	+	−	+		−	−	+	++++

* Result reported by Brown, Frost and Shaw (1926).

† Result obtained in this laboratory.

The results, contained in table 5, were obtained with strains which were isolated from cows or milk and six of which were obtained at the time of human epidemics occurring in the same localities.

All of these strains possess fibrinolytic activity to a high degree. The organisms were considered by the investigators from whom they were received as being the cause of acute infection among

TABLE 6

Fibrinolytic activity and biochemical reactions with bovine cultures, not classified as human pathogenic strains

STRAIN	LABORATORY	SOURCE	FERMENTATION OF			pH IN 1 PER CENT GLUCOSE	HYDROLYSIS OF SODIUM HIPPURATE	FERMENTATION OF		LANCEFIELD SERIOLOGICAL CLASSIFICATION	FIBRINOLYTIC TEST	
			Lactose	Mannitol	Salicin			Sorbitol	Trehalose		Fibrinogen*	Plasma†
$K_{106}G$	Lancefield	Normal	+	−	+	4.7	−	+	−	Group C	−	−
$K_{106}H$	Lancefield		+	−	+	4.8	−	+	−	Group C	−	−
$K_{106}J$	Lancefield		+	−	+	4.7	−	+	−	Group C	−	−
K_{106}	Lancefield	Mastitis	+	−	+	4.7	−	+	−	Group C	−	−
V_9	Lancefield		+	−	−	4.4	+	−	+	Group B	−	−
V_9	Lancefield		+	−	−	4.4	+	−	+	Group B	−	−
K_{107}	Lancefield	Mastitis	+	−	−	4.4	+	−	+	Group B	−	−
B_{43}	Lancefield	Acute mastitis	+	−	−	4.5	+	−	+	Group B	−	−
B_{113}	Lancefield	Mastitis	+	−	−	4.4	+	−	+	Group B	−	−
B_{115}	Lancefield	Mastitis	+	−	−	4.6	+	−	+	Group B	−	−
B_{116}	Lancefield	Mastitis	+	−	−	4.4	+	−	+	Group B	−	−
M_{116}	Lancefield		+	−	−	4.5	+	−	+	Group B	−	−
K_{127}	Lancefield		+	−	+	4.5	+	−	+	Group B	−	−
K_{128}	Lancefield	Certified milk	+	−	−	4.8	−	+	+	Group E	−	−
K_{129}	Lancefield	Certified milk	+	+	−	4.6	−	+	+	Group E	−	−
K_{131}	Lancefield	Certified milk	+	−	−	4.6	−	+	+	Group E	−	−
39	Brown	Milk	+	−	+	5.0	−	+	−		−	−
K.Gp.14a	Brown	Milk	+	+	+	4.7	− or ±	+	+		−	−
C_{18}	Brown	Milk	+	−	−	4.7	−	−	+		−	−
E_1	Edwards	Mastitis	+	−	+	5.0	−	+	−	Group C	−	−
E_2	Edwards	Mastitis	+	−	+			+	−	Group C	−	−
E_3	Edwards	Mastitis	+	−	+			+	−	Group C	−	−
E_5	Edwards	Mastitis	+	−	+	5.1	−	+	−		−	−
E_7	Edwards	Mastitis	+	−	+	4.7	−	+	−		−	−
E_4	Edwards	Mastitis	+	−	+			+	−		−	−
E_9	Edwards	Mastitis	+	−	+	4.7	−	+	−	Group C	−	−
E_{10}	Edwards	Mastitis	+	−	+			+	−		−	−
E_{13}	Edwards	Mastitis	+	−	+	4.7	−	+	−		−	−

* Human fibrinogen coagulated with human thrombin.

† Human plasma coagulated with $CaCl_2$.

TABLE 6—*Concluded*

STRAIN	LABORATORY	SOURCE	FERMENTATION OF			pH IN 1 PER CENT GLUCOSE	HYDROLYSIS OF SODIUM HIPPURATE	FERMENTATION OF		LANCEFIELD SERIOLOGICAL CLASSIFICATION	FIBRINOLYTIC TEST	
			Lactose	Mannitol	Salicin			Sorbitol	Trehalose		Fibrinogen*	Plasma†
25261	Edwards	Placenta abortion	+	−	+	4.7	−	+	−	Group C	−	−
25505	Edwards	Brain, calf septicemia	+	−	+	4.7	−	+	−	Group C	−	−
28117	Edwards	Brain, calf septicemia	+	−	+	4.7	−	+	−	Group C	−	−
Per	Edwards	Peritonitis fatal	+	−	+	4.8	−	+	−		+	+
126 Ut A	Edwards	Metritis fatal	+	−	+	4.7	−	+	−		+	+
E_{12}	Edwards	Mastitis	+	−	+	4.7	−	+	−		+	+
Beauty	Brown	Mastitis	+	−	+	4.9	−	+	−		+	+
Blackie	Brown	Mastitis	+	−	+	4.8	−	+	−		+	+

the population drinking the contaminated milk. It is interesting to note that strain C_2b has been in artificial cultivation for twenty-one years.

The results obtained with the remaining thirty-six bovine strains are given in table 6. These cultures act in biochemical reactions and in serological classification as animal strains.

From the table it may be noted that thirty-one of the thirty-six strains are incapable of causing fibrinolysis. Five of the strains are classified as weakly positive. Repeated tests have been performed with these cultures. Occasionally no liquefaction has occurred; at other times lysis was effected, either partially or completely, in from ten to twenty-four hours. The final conclusion has been, therefore, that the strains do elaborate a small amount of fibrinolysin. In thirty-eight strains from "veterinary tissues" Madison (1934) found thirty-five to be non-fibrinolytic and three to be weakly active.

At the present time it is not possible to interpret the results

obtained with the slowly acting cultures. Studies are now in progress concerning the effect of environmental factors in the production of fibrinolysin by the bacterial cells, and on the occurrence of variants among the culture population.

The data in tables 5 and 6 indicate that the presence of fibrinolytic activity coincides, in general, with the biochemical reaction of human strains of beta hemolytic streptococci, whereas the non-lytic cultures behave, in other respects, as animal strains. Lysis accompanies with great frequency the selective action on sorbitol or trehalose of the strains which fulfill the conditions defined by Edwards for classification. The representatives of the serological groups, which constitute the subdivisions of the animal strains, were inactive against fibrin regardless of whether they belonged to group B, C, or E.

It is interesting to note in passing that, of the ten group B strains, eight fermented lactose, but failed to attack mannitol or salicin. This selectivity in fermentative reactions conforms to the *anginosus* group of Holman (1916).

Strains V 9 and B 112 were double zoned hemolytic streptococci (Brown, 1934).

The final results with the two hundred and seventeen strains of hemolytic streptococci, when tabulated with respect only to origin and fibrinolytic activity, are as follows:

Human strains	157
Fibrinolytic	154 (98.1 per cent)
Negative	3 (1.9 per cent)
Animal strains	53
Fibrinolytic	6 (11 per cent)
Negative	47 (89 per cent)

When the test was applied to the problem relating to the human pathogenicity of hemolytic streptococci isolated from milk or cows, the results were as follows:

Strains of Streptococcus epidemicus (human pathogens)	7
Fibrinolytic	7 (100 per cent)
Average time required for dissolution: 18 minutes	

Strains bovine type	36
Negative	31 (86.1 per cent)
Fibrinolytic	5 (13.9 per cent)

Average time required for dissolution: 12 hours

DISCUSSION

The results, presented in this communication, represent an attempt to correlate the fibrinolytic activity with other biological characteristics and biochemical reactions of beta hemolytic streptococci. With the selected strains, which were employed, and under the experimental conditions, which have been described in detail, the capacity of the organisms to attack human fibrin bore a definite relationship to human pathogenicity. The preponderant majority of strains isolated from cases of infection were fibrinolytic. Furthermore, streptococci from patients having severe, spreading infections often had maximal lytic potency, whereas strains from milder disorders tended to be weaker. The non-fibrinolytic strains consisted of a group which were not associated with illness or were derived from animals. These strains are, in all probability, not pathogenic for man.

Although Hadfield, Magee, and Perry (1934), and Madison (1934) did not find fibrinolysis to be as constantly associated with human strains as has been the experience in this laboratory, the authors just mentioned did report that the more potent strains came from the more serious disorders, and that the animal strains, which they tested, were inactive or very slowly lytic.

The identification of cultures of hemolytic streptococci, which are either an actual or potential menace to man, and their separation from other streptococci, which come from a variety of sources and are probably not capable of producing human disease, is a well-recognized problem. A review of the literature reveals the advances which have come from the investigations of those who have analyzed the biological and biochemical characteristics of streptococci. However, with the exception of the serological classification recently devised by Lancefield, no single test seems adequate in the evaluation of the culture brought into question. Furthermore, the properties of bacteria which endow them with

virulence consist of a complicated mosaic of attributes which include insusceptibility to phagocytosis (Hare, 1934), capacities to survive and multiply in the tissues, production of toxin, and other methods of causing tissue damage. It becomes necessary, therefore, in studying the mechanism of bacterial pathogenicity to consider both qualitatively and quantitatively, the several properties possessed by the organisms and to evaluate them with respect to the type and degree of host immunity. The particular activity of hemolytic streptococci, to which this presentation is limited, concerns a single specific bacterial property, which acts upon and alters a particular type of tissue, namely fibrin.

CONCLUSIONS

There is a relationship between the infectivity of beta hemolytic streptococci for man and the fibrinolytic property of the cultures.

The determination of the presence or absence of fibrinolysin in the cultures is a helpful procedure in separating human pathogenic strains from others which probably do not cause human disease.

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THE MORGAN BACILLUS

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The isolation by Morgan (1906) of a previously undescribed bacillus—"Morgan's bacillus Type I"[1]—from the stools of 28 out of 58 infants with summer diarrhea was followed by other studies[2] which were thought to indicate that this organism bears a causal relation to diarrhea in infants and young children. The causation of adult diarrhea and dysentery has also been attributed to this organism, particularly by French authors and by students of dysenteric infections during the Great War.[3] Phalen and Kilbourne (1910) considered that dysentery in the Philippines was due in part to the Morgan bacillus; this was "the only type of dysentery" encountered in certain regions. Later investigators have found the Morgan bacillus in a variety of pathological con-

[1] Several other bacillary "types" were described by Morgan, but they have not been as commonly reported by later investigators, and "Type I" has by common consent become "Morgan's bacillus," "B. Morganii," or "Salmonella morganii." "B. morgani" is used synonymously with "B. Morgan No. 1" in the 3d edition of the Catalogue of the National Collection of Type Cultures, London, 1931.

Waaler (Jour. Bact., 1931, **22**, 261) has recently reported the presence of Morgan's Type XII in five cases of infection of the urinary tract.

[2] Morgan, 1907; Morgan and Ledingham, 1908–9; Alexander, 1911–12; Lewis, 1912–13.

[3] Tribondeau and Fichet, 1916; Archibald, Hadfield, Logan and Campbell, 1916, (who observed that in a series of diarrheas connected with the advent of sandstorms "Morgan's bacillus was the only known pathogenic organism isolated"); Delille, Paisseau, and Lemaire, 1916; Thomson and Mackie, 1917 (who studied dysentery in Egypt and state that 5 out of 30 cases "very thoroughly examined" showed *B. Morgani* "present in large numbers"); Pirie, 1917 (who worked on East African dysentery—note, however, the divergent cultural characters of the African "Morgan bacilli" in the American Type Culture Collection, Nos. 973–978); Douglas and Colebrook, 1920; Rey, 1920; Besson and deLavergne, 1921; Dopter, 1921.

ditions: chronic discharging wounds (Whittingham, 1919); ulcerative colitis (Thjötta, 1920); fatal septicemia (Magath and Jackson, 1925); pyelitis (Riding, 1927); fatal cholecystitis (Thjötta, 1928); pyelitis and colitis (d'Aunoy, 1929); enteritis associated with mastoiditis in infants (Dick, Dick and Williams, 1928); and summer diarrhea in children (Mackenzie and Batt, 1930). Stewart (1928) has claimed that the Morgan bacillus is commonly present in the intestinal tracts of patients with mental disease and that its elimination from the bowel (through vaccination) is followed "by improvement in bodily health and possibly improvement of the mental condition.

On the other hand, its pathological significance, especially in gastrointestinal illness, has been questioned by several writers. Davidson (1920, 1922), who made a careful study of dysentery in Baltimore, Maryland, and Birmingham, Alabama, considers it "highly improbable that B. Morgan No. 1 is of etiological importance in diarrhea." Davidson and also Tenbroeck and Norbury (1915) point out that this bacillus does not agglutinate with the serum of patients in whose stools it is found and that it is not infrequently present in the stools of normal persons and in animals. Gardner (1929), in a recent critical summary, decides that "there is no conclusive evidence that Morgan's bacillus is the cause of intestinal disturbance or any other disease in man."

An etiological relation of Morgan's bacillus to typhoidal or paratyphoidal diseases in the United States has recently been suggested anew by the work of Havens and Ridgway (1929a, 1930) who state that "In a series of 49 cases [in Alabama], clinically resembling paratyphoid fever, *B. Morgani* was isolated from the feces in all the cases, from the blood in six cases and from the urine in 11." It was thought by Havens to be particularly significant that, while agglutination tests with other organisms were invariably negative, agglutinins for the strain recovered from each patient were present in that patient's serum in titers ranging from 1:40 up to 1:2560.

In the course of studies on gastrointestinal disease the writers have not infrequently isolated bacilli that resembled more or less closely the "*B. Morgani*" type. Notes made on these organisms, supplemented with cultural and serological tests upon strains

from type culture collections and other sources, are here recorded as a contribution to the study of this group.

The salient features of Morgan's bacillus mentioned by most writers are: (1) gas production in glucose, levulose and galactose; (2) no fermentation of lactose, sucrose, mannitol or dulcitol; (3) indol production; (4) motility usually present, sometimes absent. It is evident, however, that such characters by themselves are insufficient for purposes of present-day bacterial identification.

Examination of the published descriptions of Morgan's bacillus reveals considerable lack of uniformity and completeness. Some features may be briefly noted.

Lactose. Although Morgan's bacillus is characterized by practically all writers as a non-lactose-fermenter, many observers have failed to specify the period of time over which their observations have extended. Among others Fleming (1919), Tribondeau and Fichet (1916), Havens and Ridgway (1929b), Kligler (1919), Lovell (1929), and Mackenzie and Batt (1930) make no statement as to the length of time their cultures were incubated. Morgan (1906) apparently incubated his lactose cultures for three days at 37°C.; Dudgeon (1919) states that final readings were made on the fifth day and Levine (1925), in what is perhaps the most extensive comparative study of the group yet reported, states that "acid and gas formation was recorded after forty-eight hours at 37°C." Unfortunately, some writers have not made it clear whether in recording "non-fermentation of lactose" they mean the absence of gas production alone, or the absence of both acid and gas production.

The unsatisfactory nature of our knowledge about this group of organisms is further accentuated by such articles as that by Dungal (1927), who includes under the name of B. metacoli or Morgan's bacillus seven strains, all giving different fermentation reactions and all but one of which are set down as producing a small amount of gas in lactose.

The practical impossibility of correlating and evaluating imperfect descriptions of bacteria is well illustrated by the nature of the statements about the lactose-fermenting power of Morgan's bacillus. Even in those instances where the period of incubation is specified, as by Levine (1925), the observations have not, as a

rule covered a period long enough to give adequate information. It is common knowledge that in deranged conditions of the alimentary tract there are present many bacteria which are slow in fermenting lactose and often give no sign of either gas or acid production until after a week or more of incubation. Colonies of these late lactose-fermenters may be picked as "suspicious" non-lactose-fermenters from plates of Endo or eosin-methylene blue medium, and when the transplants do not show any signs of fermenting lactose over a period of several days they are frequently classed by the observer as "Morgan-like" or "paratyphoid-like" bacilli. Unless lactose tubes are kept under observation as a preliminary test for at least two weeks, a large amount of useless cultural and serological work may be expended on these late lactose fermenters.

The uncertainty about lactose fermentation shown by these facts vitiates much of the comparative work that has been done with cultures of the "Morgan bacillus." In some instances late lactose fermenters have been apparently confounded with the non-lactose-fermenting "true" Morgan bacillus.

Sucrose. Most descriptions of the Morgan bacillus[4] agree in stating that sucrose is not fermented. The criticism already made with respect to the time limitations on lactose fermentation applies also to sucrose. Unless an adequate period of incubation is allowed to elapse (at least fourteen days), inability to ferment sucrose cannot be regarded as established. Two of the six "Morgan" strains from the American Type Culture Collection which fermented lactose fermented sucrose also (acid in seven days); the strain from the Lister Institute Collection of "Morgan" strains which fermented lactose also fermented sucrose (acid and gas, fourteen days). Coleman (1931) isolated from a parrot a sucrose-fermenting bacillus identified as *B. Morganii*. This organism agglutinated with serum prepared from the standard Lister Institute strains When examined later by other observers (Havens and Irwin, 1932) it was not found to possess sucrose-fermenting power.

[4] Morgan, 1906, 1907; Tribondeau and Fichet, 1916; Thjötta, 1920, 1928; Mackenzie and Batt, 1930; Gardner, 1929; Havens and Ridgway, 1930; Kligler, 1919; Levine, 1925; Bergey, 1930.

Gelatin. All statements agree that gelatin is not liquefied by authentic strains of the Morgan bacillus.

Indol. There is also practically unanimity that the Morgan bacillus produces indol. Levine (1925), however, states that 2 out of his 31 strains did not form indol.

H_2S formation. There is general agreement that sulfuretted hydrogen is produced.[5]

Motility. The majority of investigators state that the Morgan bacillus is actively motile; a few report it as slightly motile;[6] others state that both motile and non-motile varieties exist. The lack of uniformity of conditions under which motility is observed impairs the value of motility as a diagnostic character.

Other carbohydrates. Salicin: negative.[7] Sorbitol: negative.[8] Rhamnose: negative.[9] Maltose: negative;[10] positive.[11] Xylose: negative.[12] Arabinose: negative.[13] Dulcitol: negative.[14] Trehalose: negative.[15] Inositol: negative.[16] Mannitol: negative;[17] positive.[18] Galactose: positive.[19] Levulose: positive.[20] Adonitol: negative.[21] Dextrin: negative.[22] Glycerol: positive.[23] Raffinose: negative.[24]

[5] Thjötta, 1920, 1928; d'Aunoy, 1929; Levine, 1925; Bergey, 1930.
[6] Thjötta, 1920, 1928; d'Aunoy, 1929.
[7] Kligler, 1919; Levine, 1925; Bergey, 1930.
[8] Havens and Ridgway, 1929; Levine, 1925.
[9] Havens and Ridgway, 1929; Kligler, 1919; Levine, 1925.
[10] Thjötta, 1920, 1928; Mackenzie and Batt, 1930; Kligler, 1919; Levine, 1925.
[11] Besson and deLavergne, 1921; Bergey, 1930.
[12] Mackenzie and Batt, 1930; Kligler, 1919; Levine, 1925 (29−, 2+).
[13] Kligler, 1919; Levine, 1925; Bergey, 1930.
[14] Mackenzie and Batt, 1930; Gardner, 1929; Kligler, 1919; Levine, 1925.
[15] Levine, 1925 (29−, 2+).
[16] Weldin, 1927; Bergey, 1930.
[17] Thjötta, 1920, 1928; Mackenzie and Batt, 1930; Gardner, 1929; Havens and Ridgway, 1929; Kligler, 1919; Levine, 1925; Bergey, 1930.
[18] Besson and deLavergne, 1921.
[19] Besson and deLavergne, 1921; d'Aunoy, 1929; Mackenzie and Batt, 1930; Kligler, 1919; Levine, 1925 (30+, 1−); Bergey, 1930.
[20] Besson and deLavergne, 1921; d'Aunoy, 1929; Mackenzie and Batt, 1930; Kligler, 1919; Levine, 1925, Bergey, 1930.
[21] Havens and Ridgway, 1929; Levine, 1925 (30−, 1+); Bergey, 1930.
[22] Mackenzie and Batt, 1930; Weldin, 1927; Bergey, 1930.
[23] Mackenzie and Batt, 1930; Bergey, 1930.
[24] Weldin, 1927.

On the basis of published descriptions Morgan's bacillus might be briefly characterized as a Gram-negative rod, fermenting glucose, levulose and galactose with gas production; not fermenting lactose, sucrose, salicin, arabinose, dulcitol, mannitol; usually without action on xylose, trehalose, adonitol;[25] indol produced abundantly; H_2S formed; gelatin not liquefied; active moti ity usually observed.

Stock collection strains. Cultures designated as Morgan's bacillus ("*Salmonella Morganii*," "*B. Morganii*," "*B. Morganii* No. 1") were received from the American Type Culture Collection, and from the National Collection of Type Cultures (Lister Institute); a number of recently isolated strains were also sent us by the late Dr. Leon C. Havens of the Alabama State Board of Health.

In this study we have ruled out all cultures producing acid or gas in lactose or sucrose within seven days. Six of the eight cultures (Nos. 973 to 978)[26] received from the American Type Culture Collection produced acid (in three instances with gas formation) in lactose; one of the 16 cultures (No. 2817) from the National Type Culture Collection produced acid and gas in lactose and sucrose; two of the six Havens cultures produced only acid (no gas) in glucose and two others produced acid promptly in sucrose.

The 19 cultures were submitted to further tests, as shown in table 1. Culturally, they seemed at first to constitute a homogeneous group corresponding to the characterization given above. Repeated fermentation tests, however, showed differences, especially when the period of observation continued for more than fourteen days.

One strain occasionally showed late fermentation (after fourteen days) in lactose, and four strains in sucrose;[27] acid also ap-

[25] Statements with respect to other carbohydrates than those named are contradictory or rest on the testimony of a single observer.

[26] These came originally from Pirie, South African Institute for Medical Research, Johannesburg, and were isolated by him during the War, 1916–18. Cf. Jour. Hyg., 1917, **15**, 565.

[27] Similar results have been obtained by Havens and Irwin (1932) who confirmed the writers' observation on the occasional fermentation of sucrose by several strains of the Morgan bacillus.

peared in some arabinose tubes after fourteen to twenty-one days; one strain fermented trehalose with production of acid and gas; five strains did not produce H_2S; seven strains produced slight acid (fourteen days) in sorbitol broth and there were some other variations.

Definite variation was shown in fermentation power by one and the same culture. Two of the strains giving slight acidity in sucrose after fourteen days were grown in nutrient broth kept at 22°C., transferred three times at two-day intervals, then plated, two colonies picked to agar slants and, after twenty-four hours growth at 37°C., again inoculated into sucrose broth. In both instances, acid appeared as early as the seventh day and there was also gas formation within fourteen days, although numerous previous tests with these strains had never shown gas production in sucrose media. In a word, the biochemical reactions of these organisms were neither uniform nor stable.

Agglutination. Although a number of investigators[28] have observed the presence of specific agglutinins for the Morgan bacillus in the blood of patients with diarrhea, the agglutinative action is generally limited to the strain isolated from the patient himself, other Morgan bacillus strains being little, if at all, affected. The antigenic heterogeneity of "Morgan bacillus" strains is further attested by practically all observers who have prepared specific sera by inoculating rabbits with strains from various sources.[29] Kligler (1919) found that 17 strains yielded 10 distinct antigenic types without any correlation as to source; Mackenzie and Batt (1930) found that 4 strains represented 3 antigenic types.

The 17 strains from type culture collections here studied have shown similar diversity (table 2). Since the results obtained are in complete accord with those of previous investigators[30] as

[28] Alexander, 1911–12; Thjötta, 1928; Havens and Ridgway, 1929; Magner, 1916.

[29] Alexander, 1911–12; Lewis, 1912–13; Thjötta, 1920; Mackenzie and Batt, 1930; Kligler, 1919; Dean, Adamson, Giles and Williamson, 1917; Castellani and Douglas, 1932.

[30] Coleman, (1931) for example, has recently reported the isolation of 20 cultures with the morphological and cultural characteristics of B. Morganii, none of which agglutinated with the serum prepared with a standard strain (Lister Institute). A culturally different strain (fermenting sucrose) was, however, agglutinated by the serum.

TABLE 1

	SOURCE AND DATE OF ISOLATION	GLUCOSE	LACTOSE	SUCROSE	LEVULOSE	GALACTOSE	MANNITOL	GELATIN	INDOL	BALITIN	SORBITOL	RHAMNOSE	MALTOSE	XYLOSE	ARABINOSE	DULCITOL	H_2S PRODUCTION	TREHALOSE	INOSITE	TARTRATE MEDIUM
A.T. 79	Old type culture from Amer. Mus. Nat. Hist. Source unknown	AG[3]	–	a[14]	AG	AG[3]	–	–	+	–	–	–	–	–	a[21]	–	+	–	–	a
A.T. 746	From Castellani 1918	AG[3]	–	a[16]	AG	AG[3]	–	–	+	–	a[14]	–	–	–	–	–	+	–	–	a
N.C. 232	B. Morganii No. 1 Spencer	AG	–	–	AG	AG	–	–	+	–	–	–	–	–	–	–	+	–	–	a
N.C. 234	B. Morganii No. 1 Fly	AG	–	–	AG	AG	–	–	+	–	a[14]	–	–	–	–	–	+	AG	–	a
N.C. 235	Original Morgan culture summer diarrhea 1906	AG	–	–	AG	AG	–	–	+	–	a[14]	–	–	–	–	–	+	–	–	a
N.C. 417	Amer. Mus. Nat. Hist. Stool of infant. Providence, R. I.	AG	–	–	AG	AG	–	–	+	–	–	–	a[14]	–	–	–	+	–	–	a
N.C. 679	B. Morganii No. 1 Morgan	AG	a[14]	a[14]	AG	AG	–	–	+	–	–	–	–	–	–	–	–	–	–	a
N.C. 1707	Epidemic of diarrhea among children, Hebron, Palestine, 1923	AG	–	–	AG	AG	–	–	+	–	–	–	–	–	–	–	+	–	–	a
N.C. 1708		AG	–	–	AG	AG	–	–	+	–	–	–	–	–	–	–	+	–	–	a
N.C. 1709		AG	–	–	AG	AG	–	–	+	–	–	–	–	–	–	–	+	–	–	a
N.C. 2814	Squirrel monkey. London Zool. Gardens	AG	–	–	AG	AG	–	–	+	–	–	–	–	–	–	–	–	–	–	a
N.C. 2814A	Abyssinian monkey. London Zool. Gardens	AG	–	–	AG	AG	–	–	+	–	a[14]	–	–	–	a[14]	–	+	–	–	a
N.C. 2814B	Orang-utan	AG	–	–	AG	AG	–	–	+	–	–	–	–	–	a[21]	–	+	–	–	a
N.C. 2815	Green-billed toucan. London Zool. Gardens	AG	–	–	AG	AG	–	–	+	–	–	–	–	–	–	–	+	–	–	a
N.C. 2816	False moccasin. London Zool. Gardens	AG	–	–	AG	AG	–	–	+	–	a[14]	–	a[14]	a[21]	a[14]	–	+	a[14]	–	a
N.C. 2818	Pine snake. London Zool. Gardens	AG	–	–	AG	AG	–	–	+	–	a[14]	–	–	–	a[14]	–	+	–	–	a
N.C. 3069	Pus—Lister Inst. 1929 "Hemolytic"	AG	–	–	AG	AG	–	–	+	–	a[14]	–	–	–	a[14]	–	+	–	–	a

H. 750	Feces of patient with severe diarrhea, 1930	AG	−	a^{14}	AG	AG	−	−	+	−	−	−	−	−	−	−	+	−	−	a
H. 861	Spinal fluid, fatal meningitis, 1932	AG	−	−	AG			−	+	−	−	−	−	−	−	−	−	−	+	a^4
P.R. 26	Enteritis—suspected food poisoning	AG	−	−	AG	AG	−	−	+	−	−	−	−	−	−	−	−	−	−	a
P.R. 27	Enteritis—suspected food poisoning (another case)	AG	a^{14}	−	AG	AG	−	−	+	−	a^{14}	−	−	−	−	−	+	−	−	a
P.R. 69	Suspected typhoid	AG	a^{14}	a^{14}	AG	AG	−	−	+	−	−	a^{14}	a^7	a^{31}	−	−	+	−	−	a
P.R. 77	Diarrhea (infant of 9 months)	AG	−	−	AG	AG	−	−	+	−	−	−	−	a^2	−	−	+	−	−	a
P.R. 146	Diarrhea	AG	−	−	AG	AG	−	−	+	−	−	−	−	a	a	−	−	−	−	a
P.R. 171	Enteritis—suspected food poisoning	AG	−	−	AG	AG	−	−	+	−	a^{14}	−	−	−	−	−	−	−	−	a

A.T. = American Type Culture Collection.
N.C. = National Collection of Type Cultures, Lister Institute.
H. = Strains received from the late Dr. L. C. Havens.
P.R. = Strains isolated by the writer from patients in Puerto Rice.

regards antigenic heterogeneity, it did not seem worth while to make absorption tests with specific sera. In order to determine the possible agglutinability of allied organisms by Morgan bacillus serum, 14 strains of indol-producing non-lactose-fermenting bacilli were tested with the No. 417 and No. 77 sera. All of these

TABLE 2

Results of agglutination tests

TEST SUSPENSION	SERUM	
	PR 77	NC 417
AT 79		1000
AT 746	200	
NC 232		
NC 234		
NC 235	100	
NC 417		1000
NC 679	200	
NC 1707		500
NC 1708		500
NC 1709		500
NC 2814		500
NC 2814B		
NC 2815		
NC 2816		
NC 2818		
NC 3069		
Havens 750	500	
Havens 861		
Puerto Rico 26	200	
Puerto Rico 27	200	
Puerto Rico 69		
Puerto Rico 77	1000	
Puerto Rico 146	100	
Puerto Rico 171	500	

strains produced gas in glucose broth and did not ferment lactose or sucrose, but did differ in various other fermentative characters from the typical Morgan bacillus. These strains were obtained from different sources: 3 of them were sent us from various collections under the name of Morgan bacillus; the other 11 were isolated by us from diarrheal stools. Twelve of the 14 did not

agglutinate with either Morgan serum in 1:40 dilution. The 2 remaining strains were derived from a patient with enteritis in Puerto Rico; one was agglutinated by both of the Morgan sera in 1:40 dilution, but not higher; the other was agglutinated 1:40 by the 417 serum and did not agglutinate with the 77 serum.

Freshly isolated strains. In the course of studies by the writers on stools obtained during outbreaks of suspected food poisoning, non-lactose fermenting indol-producing bacteria have been isolated from time to time and subjected to further study. It may be noted that all these strains of Morgan bacilli were obtained from diarrheal conditions; none were found in healthy persons or in the stools of monkeys or other animals. Six strains possessing the biochemical properties usually regarded as characteristic of the Morgan bacillus were isolated by us at the School of Tropical Medicine, Puerto Rico.[31] These 6 strains agreed culturally with the 17 strains from Type Culture Collections which have already been described; 3 of them were from feces in suspected food poisoning cases, one from a case of suspected typhoid, two from enteritis of unknown origin (one in an infant of nine months). Several of the strains, like the Type Culture strains, occasionally produced late acid (fourteen days) in lactose, sucrose, sorbitol or maltose broth (table 1).

DISCUSSION

1. Differential characters of Morgan's bacillus

Much of the current uncertainty about the significance of Morgan's bacillus is due to the incompleteness of the descriptions given by some of the observers who have reported finding this organism in pathological and normal conditions. The bacteriological details necessary for identification are completely lacking in some instances and in others are so meager that a measure of doubt must remain. Particularly with respect to lactose fermentation, many of the published descriptions are inadequate in that they do not specify the observance of a period of incubation

[31] We are indebted to Dr. E. B. McKinley, the Director of the School, for many courtesies.

long enough to determine this quality. Since many indol-producing bacteria isolated from the intestinal contents of man and other animals do not show signs of fermenting lactose within a period of one, two or three days, but do manifest active fermentation when incubated for a week or more, we are inclined to believe that these late lactose fermenters have sometimes been set down by observers as "Morgan bacilli," particularly since a number of cultures sent from the large type collections of bacteria, as well as cultures from individual investigators, have proved to be of this kind.

So far as can be determined by the published descriptions of Morgan's bacillus and by our own observations on 19 cultures identified by various investigators as Morgan's bacillus together with 6 other similar cultures isolated by us in Puerto Rico, the following characters seem to be relatively constant: a Gram-negative rod, actively motile,[32] not liquefying gelatin, producing indol abundantly, forming H_2S, fermenting glucose, levulose, galactose with acid and gas production, not fermenting lactose, sucrose, mannitol, salicin, sorbitol, rhamnose, maltose, xylose, arabinose, dulcitol, trehalose and inositol except that occasionally some strains after seven to fourteen days growth produce acid (never gas) from one or more of these carbohydrates. None of the 25 strains that we have examined, however, have ever produced acid from mannitol, dulcitol, inositol or salicin. All 25 strains have given a positive reaction in tartrate medium (Jordan and Harmon, 1928), resembling in this respect typhoid and colon bacilli and nearly all the other members of the intestinal group except *Salmonella paratyphi-B*.

2. Agglutination

Practically all experimenters are agreed that no demonstrable antigenic unity exists within the group of Morgan bacilli. Our own results (table 2) are in accord with these observations.

[32] A number of the strains examined by us showed no motility when grown at 37°C. whereas cultures at 20° were actively motile. Some of the statements that the Morgan bacillus is non-motile may be due to the fact that only cultures grown at 37°C. were examined.

Havens and Irwin (1932) observed an antigenic change coincidental with the acquisition of sucrose fermentation, "no cross-agglutination" occurring "between the sucrose-fermenting and the nonsucrose fermenting strains from the same culture."

3. Occurrence and distribution

Bacteria of the "non-lactose-fermenting" group identified by the finder as "Morgan bacilli" have been sometimes reported as present in the stools of normal persons.[33] In some instances specific identification appears to have been based on insufficient data so that it is difficult to evaluate certain of the published reports. In general, Morgan's bacillus seems to have been found more often in Great Britain (Topley and Wilson, 1929) than on the continent of Europe or in North America. Pirie (1917) reported finding Morgan's bacillus in East African dysentery, but the cultures deposited by him in the American Type Culture Collection do not resemble the standard strains of the organism. Morgan's bacillus has been reported not only in normal human beings and in patients with diarrhea or dysentery, but in the intestines of various animal species. Morgan and Ledingham (1908–9) found it in cows' feces. Lovell (1929) isolated it from a number of animals in the London Zoological Gardens (monkey, pine-snake, green-billed toucan, etc.). The cultures of the Lovell bacilli obtained from the Lister Institute Collection have mostly conformed to the standard known strains. Wilson (1927) observed a spontaneous epidemic among mice which was apparently caused by the Morgan bacillus. Flies and cockroaches caught in infested houses have been reported as harboring the Morgan bacillus.

Our own platings in Puerto Rico of 40 to 50 stools of normal adults, and in Panama of 200 to 250 stools of normal children, did not yield any "true" Morgan bacilli possessing the characters outlined in this paper, although slow lactose-fermenters were frequently found. Similarly, an examination in Panama of the

[33] Morgan and Ledingham, 1908–9; Davison, 1920, 1922; Tenbroeck and Norbury, 1915; Logan, 1914; Eyre and Minett, 1909.

feces of recently captured animals (several species of South American monkeys, opossum, armadillo, bat) did not result in the isolation of the Morgan bacillus. Platings of the stools of 29 healthy persons and 97 persons with intestinal disorders by one of us (McBroom, 1930) in Chicago did not yield any Morgan bacilli. The relatively frequent occurrence of this organism in Great Britain is of interest.

4. Classification

The position of Morgan's bacillus with reference to other intestinal bacteria has been much debated. Winslow, Kligler and Rothberg (1919) express their opinion about this organism as follows: "It may perhaps represent an extreme variant of the variable *B. dysenteriae* group but we have considered it with the paratyphoids on account of its gas production." In Bergey's classification (1930) it is placed in the genus Salmonella, and Castellani and Douglas (1932) also regard it as belonging with the Salmonella type. Thjötta (1928), however, maintains that it belongs properly in the genus Escherichia or *B. coli* group and should be named *Bact. metacoli* as it was called by Danish investigators. In this opinion he is supported by d'Aunoy (1929). On the other hand, Havens and Mayfield (1930) consider that their own observations favor the Salmonella classification. In the System of Bacteriology of the Medical Research Council (Gardner, 1929) Morgan's bacillus is included in the dysentery group of bacilli among the "paradysentery" bacilli. German bacteriologists have apparently been little interested in this organism; it is given only the briefest mention in the third edition of the Handbuch der pathogenen Mikro-organismen (Kolle, Kraus and Uhlenhuth).

The active motility of all the strains we have studied, the profuse indol production and the production of gas from glucose, levulose and galactose are characters that seem to set the Morgan bacillus apart from the dysentery bacilli. The limited action on carbohydrates and the ability to form indol, on the other hand, mark it off from the Salmonellas. We are of the opinion that so far as any specific type of Morgan bacillus can be recognized to-

day it is related much more closely to the *Bact. coli* group than to either the paratyphoid or dysentery bacilli. The tendency of some strains, generally classed as Morgan bacilli, to produce acid in lactose and sucrose,[34] the occasional production of acid in other carbohydrate media, the general and abundant production of indol, the occurrence in the animal intestine and especially the existence of many independent agglutination strains all suggest relationship to *Bact. coli*.

It should be pointed out that the strains described in this paper represent a narrow and restricted choice. Many strains of organisms carried in type culture collections as Morgan bacilli and many sent to us by independent investigators, as well as a still larger number isolated by ourselves, have been more or less arbitrarily excluded from our tabulation as departing too widely from the generally accepted criteria. A large variety of such strains have been observed and seem to form a series of connecting links with the *Bact. coli* group. It is of doubtful value to bestow specific names on these organisms at the present time, especially since variations in carbohydrate fermentation and in some other characteristics are frequently observed under cultivation.[35]

The Morgan bacillus does not seem to be a sharply defined species comparable to *Salmonella enteritidis* or *S. paratyphi-B*. It bears greater resemblance to the free-living Proteus forms or to the large group of slow-lactose-fermenters ordinarily classed with *Bact. coli*. It does not give evidence of being stabilized by long parasitism in the mammalian body in any such way as the typhoid bacillus and the Shiga bacillus are stabilized. One may express some doubt as to whether a specific limitation is justified, whether there is after all any organism so definitely and consistently marked off as to deserve the designation of *Bact. Morganii*. For the present it would seem reasonable to regard these ill-defined strains as forming part of the large and variable group of bacteria which, with the Danish bacteriologists, we may call *Bact.*

[34] In addition to our own observations (table 1), see Havens and Irwin (1932) and Coleman (1931).

[35] See, for example, Coleman (1931); Havens and Irwin (1932); and this article.

metacoli. Their pathogenic properties seem very similar to those occasionally observed among *Bact. coli* strains, the "slow-lactose-fermenters," etc.; there is little doubt that they are occasional inciters of gastrointestinal disturbance, and of suppurative processes in various organs.

SUMMARY

Many of the type culture strains labelled with the name of Morgan bacillus do not agree in significant cultural characters with the generally accepted criteria for this group. When widely variant strains are eliminated a certain residue of fairly uniform strains is left. Even among these there is far from being as close a correspondence as that between the strains of *Salmonella enteritidis* or of the Sonne type of dysentery bacillus. Antigenic uniformity is lacking.

Freshly isolated fecal strains with "Morgan bacillus" characteristics have, in our experience, been isolated only from persons suffering from some form of gastro-intestinal derangement.

The cultural and antigenic characters of Morgan bacilli seem to relate them nearly to *Bact. coli*. They may be reasonably regarded as an ill-defined division of the large and variable group conveniently known as *Bact. metacoli*. They possess perhaps somewhat greater pathogenic power for man than the "slow lactose fermenters" or true *Bact. coli* strains.

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THE PRECIPITATION OF BACTERIAL POLYSACCHARIDES WITH CALCIUM PHOSPHATE[1]

PNEUMOCOCCUS

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Many of the recent immunological studies of the pneumococcus have been concerned with the specific precipitating agent (SSS) described by Dochez and Avery in 1917, and since studied in detail by Heidelberger and Avery (1923, 1924), Heidelberger, Goebel and Avery (1925) and Heidelberger, Sia and Kendall (1930). Essentially their method of preparation consists of evaporation of the pneumococcus broth, separation of active material by repeated fractional precipitation with alcohol from neutral and acid solutions, and further purification varying with each of the three fixed types. Schiemann and Casper (1927–8), and later Saito and Ulrich (1929) prepared specific precipitating material from pneumococcus cells only, of Types I and II, by alcoholic precipitation from a sodium taurocholate solution of the organisms which had first been heated with acetic acid to remove protein. In 1930 Goebel reported modifications of Heidelberger's method, which made the technic adaptable to storing crude material made from large volumes and purifying as desired. Wadsworth and Brown (1931, 1933) prepared a "cellular carbohydrate" by the method of Schiemann and Casper except that solution was obtained by alternate freezing and thawing of the organisms. In like manner, Tillett, Goebel and

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Avery (1930), prepared the species-specific substance from both rough and smooth strains by using large quantities of alcohol for precipitation. In 1933, Dudley and Smith suggested that, for preparation from broth, the protein be first removed from the hot broth by precipitation with acetic acid, and the large volume then concentrated by evaporation. Avery and Goebel (1933) reported "acetyl derivatives" of Type I SSS prepared from both broth and cells, the former prepared by a modification of the SSS technic of Heidelberger and Avery (i.e., avoiding the use of alkali), and the latter prepared by heating autolysed cultures with acetic acid to remove protein—a method similar to Zinsser's technic (Zinsser and Parker, 1923) for preparing "residue antigens"—followed by precipitation of the carbohydrate with alcohol from acid solution.

In the above procedures in which broth was the source of the SSS, the initial concentration of the large volume was effected by means of evaporation on a steam bath. To avoid this procedure, both because of the time required and because of the possibility of some alteration in the activity of the product due to prolonged heating, a method of precipitation of the active material from the unconcentrated culture broth was sought. During a preliminary study in which various calcium salts were used as precipitants, Dr. C. H. Fiske suggested that, inasmuch as the phosphate of calcium is a selective adsorbent for complex carbohydrates, this salt might be used to advantage (Felton, 1932). Calcium phosphate was used for enzyme separation as early as 1860 by Brücke (cited by Effront, 1917). Its use for precipitation of active immunological material was advocated for the purification of diphtheria toxin first by Roux and Yersin in 1889, and later continued by Abt in 1928 and Ohyama in 1931.

Preliminary work with pneumococcus filtrate in this laboratory demonstrated that in appropriate concentration and at optimum hydrogen ion concentration, either calcium or strontium phosphate when formed *in situ* will carry down most of the specific substance. The present study therefore reports the development of a practical method of preparing SSS by the use of calcium or strontium phosphate as the initial precipitant.

MEDIA

The media used were a haddock infusion broth, and a horse-meat infusion broth. In both cases, the media were prepared by Hartley's (1922) modification of Douglas' technic. Although fish broth gives a somewhat higher yield in weight and in precipitin titer of SSS, both media so prepared proved satisfactory. In general SSS production is greater the longer the period of incubation of the culture up to eight days.

DETERMINATION OF OPTIMUM CONDITIONS FOR PRECIPITATION

The method of determining optimum conditions for the precipitation of SSS depends upon three variables: calcium content; phosphorus content (in the form of phosphate); and hydrogen ion concentration. An experiment showing the results of such a study is given in table 1. The procedure was as follows: After three-day growth of Type I pneumococci (Neufeld) on beef infusion broth, most of the organisms were removed from the broth in a Sharples centrifuge. (The cells were worked up separately.) Specific serum was then found to be precipitable by a 1:256 dilution of the supernatant broth. A series of 10-cc. samples of this broth was set up in three groups as shown in the table. The reagents, all C. P., were used in the following concentrations: 11.6 per cent $Na_2HPO_4 \cdot 12H_2O$ to give 10 mgm. phosphorus per cubic centimeter; normal sodium hydroxide; and 10 per cent calcium chloride in a saturated solution of calcium hydroxide. After standing at room temperature until flocculation had begun, the precipitates were centrifugalized and dissolved in normal HCl to pH 1 (pink with phenol red). The SSS was then precipitated by the addition of three volumes of alcohol, and to hasten the precipitation, one-fourth to one-third volume of ether. After centrifugalizing, this precipitate was dissolved, neutralized and made up to 10 cc. in 0.85 per cent sodium chloride, the original volume of broth taken. Dilutions were then made for tests with serum, the results of which are shown in the table. Briefly, the protocol shows that the variable of greatest significance is hydrogen ion concentration. For, with a constant amount of calcium chloride-

calcium hydroxide solution, as little as 0.1 mgm. phosphorus per cubic centimeter of broth, in addition to that present in the

TABLE 1

Determination of optimum conditions for precipitation

Constant calcium, varying pH and phosphorus. Type I

11.6 PER CENT $Na_2HPO_4 \cdot 12H_2O$	NORMAL NaOH	10 PER CENT $CaCl_2$ IN $Ca(OH)_2$ (saturated)	pH	PRECIPITATION WITH IMMUNE SERUM DILUTED 1:5							
				Dilutions of samples							
				Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128
cc.	cc.	cc.									
0.5	0	2.5		+	+	+	+	±	−	−	−
0.5	0.2	2.5		+	+	+	+	+	−	−	−
0.5	0.4	2.5		+	+	+	+	+	+	±	−
0.5	0.6	2.5		+	+	+	+	+	+	±	−
0.5	0.8	2.5		+	+	+	+	+	+	±	−
0.5	1.0	2.5		+	+	+	+	+	+	±	−
0.2	0	2.5		+	+	+	+	±	−	−	−
0.2	0.2	2.5		+	+	+	+	+	±	−	−
0.2	0.4	2.5		+	+	+	+	+	+	+	−
0.2	0.6	2.5		+	+	+	+	+	+	+	−
0.2	0.8	2.5		+	+	+	+	+	+	+	−
0.2	1.0	2.5		+	+	+	+	+	+	+	−
0.1	0	2.5	7.0	+	+	+	±	−	−	−	−
0.1	0.1	2.5	7.4	+	+	+	+	±	−	−	−
0.1	0.2	2.5	7.8	+	+	+	+	+	±	−	−
0.1	0.3	2.5	8.3	+	+	+	+	+	+	±	−
0.1	0.4	2.5	8.9	+	+	+	+	+	+	+	±
0.1	0.5	2.5	9.6	+	+	+	+	+	+	+	±
0.1	0.6	2.5	9.6*	+	+	+	+	+	+	+	±
0.1	0.7	2.5		+	+	+	+	+	+	+	±
0.1	0.8	2.5		+	+	+	+	+	+	+	±
0.1	0.9	2.5		+	+	+	+	+	+	+	±
0.1	1.0	2.5		+	+	+	+	+	+	+	±
Control				+	+	+	+	+	+	+	+

* The remainder of the series were at pH above 9.6, i.e., maximum color with thymol blue—exact pH not tested.

original broth, is sufficient to carry down the SSS providing the pH is optimum.

Table 2 shows the effect of varying the calcium and the phosphorus content at a constant hydrogen ion concentration (pH 9). The technic of handling the precipitates was similar to that in the above experiment. Inasmuch as calcium phosphate is less soluble in calcium ions than in sodium ions, the hydroxyl ions in this experiment were furnished by calcium hydroxide instead of the sodium hydroxide used in the preceding experiment. Two in-

TABLE 2

Determination of optimum conditions for precipitation

Constant pH, varying phosphorus and calcium. Type I. Sixty-six-hour growth on fish medium; all samples made pH 9 with 10 per cent $Ca(OH)_2$.

11.6 per cent $Na_2HPO_4 \cdot 12H_2O$	10 per cent $CaCl_2$	Precipitation with immune serum diluted 1:5									
		Dilutions of samples									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
cc.	cc.										
0	0	+	+	+	+	+	+	+	±	−	−
0.05	0	+	+	+	+	+	+	+	−	−	−
0.1	0	+	+	+	+	+	+	+	+	−	−
0	1	+	+	+	+	+	+	+	+	−	−
0.05	1	+	+	+	+	+	+	+	+	−	−
0.1	1	+	+	+	+	+	+	+	+	±	±
0	2	+	+	+	+	+	+	+	+	±	±
0.05	2	+	+	+	+	+	+	+	+	±	±
0.1	2	+	+	+	+	+	+	+	+	±	±
0	3	+	+	+	+	+	+	+	+	±	±
0.05	3	+	+	+	+	+	+	+	+	±	±
0.1	3	+	+	+	+	+	+	+	+	±	−
Control.....		+	+	+	+	+	+	+	+	+	±

ferences may be made from the results of this experiment shown in the protocol. First, addition of calcium in the form of calcium chloride causes a more complete precipitation of SSS; but, with this medium at least, if sufficient phosphorus is present, 1 per cent of added calcium chloride is as effective as double or triple that amount. Second, this medium prepared from fish by Hartley's method (the same is true for media prepared from

horse meat), contains insufficient phosphorus for maximum precipitation. Only on the addition of phosphorus in concentration of 0.05 to 0.1 mgm. per cubic centimeter of broth, and under otherwise optimum conditions, is SSS obtained in the amount approaching that of the original broth.

From similar experiments it has been found that the optimum hydrogen ion concentration varies slightly with the different types: for Type I, about pH 8.6 to 9, and for Types II and III, pH 9 to 10 (or with thymol blue as indicator, a greenish blue for Type I and a definite blue for Types II and III). Too great an alkalinity however will redissolve some of the SSS from the precipitate.

PROCEDURE OF PRECIPITATION

First step—calcium precipitation

Because in general the media used in this study did not contain sufficient phosphorus to allow for complete precipitation of SSS, a preliminary test similar to the ones described above was necessary to determine the amount of disodium phosphate to be added. For our media this was usually 10 to 20 cc. of the 11.6 per cent disodium phosphate solution per liter of broth, i.e., from 0.1 to 0.2 mgm. phosphorus per cubic centimeter of broth. Furthermore, because of the results shown in table 2, a suspension of calcium hydroxide of 10 per cent concentration has been adopted to furnish the hydroxyl ions simultaneously with the calcium, without increasing the content of sodium ions. The decision as to the use of calcium chloride to increase the calcium ion content and thus decrease the solubility of calcium phosphate depends upon the medium in which the organisms are grown. Since the calcium phosphate precipitation carries down other polysaccharides such as glycogen, a high content of these inert polysaccharides in the broth may mean too great an increase of inactive material which can be separated from the SSS precipitate only with great difficulty. The lower yield obtained by the use of less calcium may be advantageous because of the ease of separation and the degree of purity of the final product.

Thus the procedure for large volumes (100 to 120 liters) has

been developed as follows: addition of phosphate in appropriate amount, followed by calcium chloride if found to be necessary, and by sufficient 10 per cent calcium hydroxide suspension to produce the desired hydrogen ion concentration.

To test whether the SSS precipitation was complete, when this technic was applied to large volumes, a 1-liter sample of the calcium supernatant broth was dialyzed to remove inorganic salts and then evaporated before a fan to the original volume of broth represented by the 1 liter of supernatant fluid taken. Serum tests have shown precipitation in about one-eighth or one-sixteenth of the dilution in which the original material precipitated, and this, in all cases tested, was a non-type-specific precipitation. One example, for instance, shows the original broth with a specific precipitate in dilution 1:128 and the dialyzed material, a non-specific precipitation in dilution 1:8. Thus, it is possible that this calcium precipitation method may leave in solution one-sixteenth to one-eighth of the specific substance along with non-specific material.

Second step—calcium removal

The possibilities for removal of calcium from this precipitate are: (1) to precipitate calcium and leave SSS in solution; (2) to precipitate SSS and leave calcium in solution; and (3) to dissolve both, and then separate by a differential precipitating agent. The first of these possibilities was tested in three ways: (*a*) with oxalic acid in the presence of either sodium or ammonium oxalate, (*b*) with ammonium sulfate and sulfuric acid, and (*c*) with sodium hydroxide. The second possibility, in the case of Type I, was tested several times with glacial acetic acid. Third, an acid, the calcium salt of which is soluble in alcohol, i.e., hydrochloric, was used for solution and alcohol for differential precipitation of SSS (leaving calcium chloride in solution). The first and second of these methods have thus far given irregular results; both serum precipitin tests and yield varied over too wide a range to consider them practical methods. Nevertheless it is possible that under optimum conditions, either would give results of practical importance.

The third procedure has, however, given consistently better results both as to yield and as to purity of the final product. Consequently this has been the method of choice. The hydrogen ion concentration necessary is pH 2 or below. In order to avoid an undue increase in the total volume, 4 N HCl has been used for this adjustment. At 4°C. more concentrated acid, although apparently without effect on the polysaccharides of Types I and III, may hydrolyze that of Type II material.

Of the possible reagents to precipitate SSS from this acid solution, ethyl alcohol has been found to be the most satisfactory. A study of the volume relationships of this and other reagents has been carried out on acid solutions of purified SSS. It was found that in the absence of neutral salts alcohol precipitates Type I SSS completely from an acid solution when two volumes are added; Type II requires three volumes; and Type III only one volume. Later work has indicated, however, that, if salts are present, more than one volume may precipitate non-specific material along with specific. These findings are a confirmation of previous work: for with five volumes of alcohol Tillett Goebel and Avery (1930) obtained non-specific material from a method by which Wadsworth and Brown (1931, 1933), using the small quantities of alcohol first advised by Heidelberger and Avery, obtained a specific precipitating substance.

Third step—purification

For purification of this crude material various means are suggested by the results of other investigators: residual protein may be separated with trichloracetic acid, and SSS obtained by subsequent alcoholic precipitation of the soluble material. Other insoluble constituents may be removed by repeated alcoholic precipitations from alternate acid and neutral solutions. A simple method of purification, which has been the most satisfactory, is dialysis of a slightly acid solution of the material for forty-eight hours, and subsequent precipitation with alcohol from a solution adjusted to the isoelectric point—for Type I pH 4.0 (according to Heidelberger) and for Type II pH 3 (according to our observations). Following such purifications, the final product is usually type-specific, and will precipitate serum when

diluted 1:2,000,000 or 1:5,000,000, or even higher in the case of Type III. Details of various methods for further purification will be reported elsewhere.

OUTLINE OF PROCEDURE

A summary of the procedure as finally developed for practical precipitation of 120-liter lots of supernatant broth of any type (I, II, or III) is as follows:

1. A preliminary test, similar to the two described above, to be carried out on each new batch of medium;

2. To the total volume add proportionate amounts of phosphate and calcium hydroxide (and calcium chloride if necessary), as ascertained above, checking pH with thymol blue.

3. After allowing to settle an hour or more, siphon and discard the supernatant fluid, and filter precipitate over night at room temperature (hardened paper, such as CSS No. 575, is satisfactory).

4. Scrape precipitate into large container and chill; From now on the procedures should be carried out in the cold (4°C.).

5. Add 4 N HCl to make about pH 2, i.e., pink with phenol red; do not remove acid insoluble at this stage.

6. Add amount of ethyl alcohol appropriate for each type to the acid solution and collect precipitate.

7. Repeat alcoholic precipitations from acid solution until the precipitate is free from calcium. Any acid insoluble material may be removed and worked up separately after the second alcoholic precipitation. Best results are obtained when the two or three successive alcoholic precipitations from acid solution are carried out as rapidly as possible. Indeed these may be done all in one day: for, if insoluble material is not first removed from the acidified solution, the salt content will be sufficient for immediate flocculation when alcohol is added. Then continue purification, as indicated by other workers, by the use of protein preciptants, or by repeated alcoholic precipitations from neutral and acid solutions, or by dialysis. Wash with redistilled acetone and dry *in vacuo*. To obtain maximum yield, precipitin tests should be made at each step of the procedure.

It should be borne in mind that inasmuch as both broth and

cultures vary appreciably, no standard procedure can be set down even for one type. Because Type II has chemical characteristics similar to glycogen, and both are precipitated with calcium phosphate, some difficulty has been experienced in separating them. The method works best with Type III. As a matter of fact, providing there is sufficient phosphorus present in the form of phosphate, 80 per cent of the Type III SSS is precipitated by simply titrating the broth with calcium hydroxide solution. The simplest method to remove the calcium and simultaneously precipitate this SSS is the use of HCl, as employed by Heidelberger for precipitation of Type III SSS. Because this specific polysaccharide is insoluble in HCl, the procedure up to the stage of purification can be carried out with not too great a loss without the use of alcohol.

A REPRESENTATIVE TYPE I PREPARATION

A representative sample of Type I SSS was prepared as follows: 91 liters of a sixty-hour growth of type I pneumococci in horse meat infusion (glucose to make 0.5 per cent and NaOH to give pH 7.6 being added after twenty-four and forty-eight hours incubation) were precipitated, after a preliminary test, by the addition per liter of broth, of 20 cc. of 11.6 per cent disodium phosphate solution and 20 cc. of 10 per cent suspension of calcium hydroxide. The precipitate was handled as described in the above outline. The weight of the moist precipitate was 2578 grams. For a first solution in acid 700 cc. of 4 N HCl were necessary; for the second, 400 cc. of normal HCl were used. The alcoholic precipitate was then suspended in water, dissolved with a minimum amount of sodium hydroxide, and precipitated with one and one-half volumes of alcohol. Two more acid and neutral solutions were precipitated with alcohol. There was then still some material insoluble in acid, from which SSS was recovered separately. The acid-soluble material was precipitated with alcohol, washed with acetone and dried *in vacuo*. The yield was 2.5 grams. Serum tests showed precipitation when the material was diluted 1:5,000,000 with Type I serum, but no precipitation with Types II and and III sera.

A MODIFIED TYPE II PREPARATION

One interesting preparation illustrative of a different method for removing calcium also shows the effect of heat on the resultant material. The calcium precipitate, obtained from 120 liters of Type II supernatant broth, was dissolved to pH 3 with 4 N HCl and excess sodium carbonate added to give maximum color with thymol blue (pH 10). The mixture was then heated at 100°C. in an Arnold sterilizer for fifteen minutes. After cooling, and centrifugalizing the insoluble material (from which SSS was recovered separately), the soluble fraction was neutralized and precipitated with two volumes of alcohol. The precipitate was twice dissolved in alkali and precipitated with one volume alcohol; then precipitated with alcohol from alternate acid and alkaline solutions twice; and finally washed with acetone and dried. The yield from 1630 grams of moist calcium precipitate was 1.4 grams. The material contained less than 0.2 per cent nitrogen, 48.9 per cent hydrolyzable sugar; precipitated only specific serum in a dilution of 1:2,500,000; but failed to produce active immunity in white mice.

DISCUSSION

Routine preparations of SSS for use in the laboratory have been made by this procedure for five years. Approximately a hundred batches each from 100 liters of broth have been so prepared. This material when diluted 1:2,000,000 to 1:5,000,000 precipitated specific immune serum; contained about 4 to 5 per cent nitrogen in Type I preparations, and little or no measurable nitrogen in Type II and Type III preparations; showed a sugar percentage after hydrolysis of 18 to 30 per cent for Type I samples and of 40 to 50 per cent for Type II samples.

The product has from the first shown characteristics different from those of the SSS of Heidelberger and Avery. In the first place, it precipitates more protein from a given immune serum: e.g., 2.8 mgm. precipitable protein were obtained in 1 cc. of serum F146, as compared with the finding of Heidelberger of 1.9 mgm. in the same serum (Felton, 1931). Second, the SSS prepared by the calcium phosphate method has consistently precipitated all

the protective antibody from immune serum. In this respect the results are the same as those obtained by Avery and Goebel with their acetyl polysaccharide. The discrepancy in this respect between Sabin's (1931) results and ours may be explained by a possible difference in the samples of SSS used. Third, from the earliest use of this method, the preparations have produced active immunity in white mice. However, in a comparative test, a sample of SSS prepared by Dr. Heidelberger by his original method was found by us to produce active immunity in white mice. Indeed, Zozaya and Clark (1933) report active immunity in white mice following injection of samples of SSS some of which were supplied by Dr. Heidelberger and others made according to his technic. Consequently the evidence seems hardly conclusive in support of the explanation of Avery and Goebel (1933) that the original preparations of SSS by the method of Heidelberger and Avery were found to be lacking in antigenic activity due to destruction of acetyl groups during the process of preparation. Confirming the work of Avery and Goebel, observations in this laboratory (Felton, 1934) have shown that Type I SSS heated at 100°C. in alkaline solution is so altered that it no longer causes complete precipitation of the protective antibody in immune serum; on the other hand, such treatment continued for one hour does not completely destroy the antigenic activity of the material. The acetyl groups may play an important part with Type I SSS, but information as to the exact rôle of this chemical group awaits further work.

It should be emphasized that calcium phosphate is not a specific precipitating agent for the polysaccharides of pneumococcus. It is, however, in part specific for complex carbohydrates when used in an alkaline reaction. At neutral reaction the precipitate obtained is largely protein rather than polysaccharide. The same general procedure outlined above has been used for isolation of polysaccharides of microorganisms other than pneumococcus, and it was found that as long as a suitable hydrogen ion concentration was maintained, a great part if not all the polysaccharide specific to the organism studied was precipitated along with other polysaccharides present in the broth.

SUMMARY

A method is outlined for preparation of the soluble specific substance of pneumococci Types I, II, and III from a broth culture by means of an initial calcium phosphate precipitation. Methods for removal of calcium are indicated, and subsequent purification follows procedures already in use.

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COMPARATIVE STUDIES OF PRESUMPTIVE TEST MEDIA FOR THE COLI-AEROGENES GROUP OF BACTERIA

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It has been agreed by most workers in water bacteriology that the plain lactose broth, usually used for presumptive tests, is not altogether satisfactory in that it gives many false positive tests. A number of modifications have been suggested to eliminate, so far as possible, these spurious positive presumptive tests. In most of the modifications dyes are used to restrain the growth of Gram-positive organisms which are the cause of many of the false positive presumptive tests obtained in plain lactose broth. Salle (1930) proposed the use of crystal violet in a phosphate-buffered, lactose, peptone broth with which he has reported excellent results. Brilliant-green lactose peptone bile is recognized by the 1933 edition of Standard Methods of Water Analysis for use in control work in water purification plants. Basic fuchsin in plain lactose broth has been advocated by Ritter (1932) and reported to have given splendid results in a large volume of tests in Kansas. Dominick and Lauter (1929) used, to good advantage, a combination of methylene blue, erythrosine and brom-cresol-purple in a lactose peptone buffered broth.

Objection has been raised by Stark and England (1932, 1933) to the use of crystal violet in presumptive tests since they found that it inhibited the growth of many strains of *Es. coli* of human origin when tested by pure culture inoculation. In commenting on the work of Stark and England, Norton (1933) points out that

[1] The writer is indebted to A. J. Wilson, J. S. Sugg, W. F. Redmond, and M. S. Wilkinson for help in the routine tests, and to Professor L. G. Willis for making determinations of oxidation-reduction potentials.

while this study has given an unfavorable report on the use of crystal violet, yet the mixed cultures found invariably in water tests might modify the results.

Horwood and Heifertz (1934) reported on comparative studies of brilliant-green lactose peptone bile, the Dominick-Lauter medium, Salle's crystal-violet broth, and Standard Methods lactose broth. They found crystal-violet broth inferior to plain lactose broth, to brilliant-green lactose peptone bile, and to the Dominick-Lauter medium.

It has been felt by the writer that additional comparative studies involving the use of crystal violet might aid in a better evaluation of its usefulness in presumptive tests. A comparison has therefore been made of crystal-violet broth, fuchsin broth, plain lactose broth and brilliant-green lactose peptone bile.

EXPERIMENTAL

Pure cultures of the Escherichia-Aerobacter group of bacteria were isolated from fresh feces of 30 individuals in apparently normal health. From one individual two specimens were obtained and from the others only one specimen each. In all, 201 strains were isolated. All were found to be Gram-negative rods, and gas producers in lactose broth. Of 200 strains tested for indol production in tryptophane broth, 188 gave positive tests.

Of the 201 strains, 183 were methyl-red positive, Voges-Proskauer negative and failed to produce turbidity in citrate broth and were thus typically *Es. coli*. Twelve strains, ten of them from a single specimen, were typical Aerobacter strains since they were consistently methyl-red negative, Voges-Proskauer positive and grew in citrate broth. The remaining 6 strains, all from one individual, were typical Citrobacter strains being methyl-red positive, Voges-Proskauer negative, and citrate positive.

The crystal violet medium was prepared in accordance with the directions given by Salle (1930) and tubed in 14 ml. amounts so that the addition of 1 ml. of a dilution of bacteria in a water blank would give a dye concentration of 1:700,000 and a composition as follows:

	per cent
Peptone	0.5
K_2HPO_4	0.5
KH_2PO_4	0.1
Lactose	0.5

Crystal violet in a final concentration of 1:700,000

Brilliant-green lactose peptone bile and plain lactose broth were prepared according to the 1933 edition of Standard Methods of Water Analysis. Fuchsin broth was the same as plain lactose broth with 0.8 ml. of a filtered saturated alcoholic solution of basic fuchsin added per 1000 ml., giving a concentration of 1:1250 of saturated alcoholic solution of basic fuchsin.

Following the method advised by Stark and England (1933) small inocula were prepared by diluting broth cultures and the approximate number of organisms per inoculum was determined by pouring agar plates. Different dilutions of the organisms tested were added to Durham fermentation tubes of lactose broth, brilliant-green lactose peptone bile, crystal-violet broth, and fuchsin broth and these tubes incubated at 37°C. Observations were made on all tubes at the twenty-fourth hour and at the forty-eighth hour. Only those inocula containing less than 50 organisms per inoculum were considered. Tests with larger inocula were repeated. Since practically all strains tested grew equally well in plain lactose broth and in brilliant-green lactose peptone bile, any test which did not show gas in both within twenty-four hours of incubation was repeated. Table 1 gives the results of the tests in crystal-violet broth. In every case the same size of inoculum was sufficient to produce 10 per cent or more of gas in both plain lactose broth and brilliant-green lactose peptone bile within twenty-four hours of incubation.

After forty-eight hours of incubation only 49 per cent of the 201 fecal coli-aerogenes organisms were found to produce as much as 10 per cent of gas in crystal-violet broth. In most of the negative tests the crystal-violet broth showed no gas whatever and in a large number showed no visible turbidity. From only 5 of the 30 persons from whom specimens were obtained did all strains produce gas in crystal-violet broth in forty-eight hours, and all

TABLE 1

Production of gas in crystal violet broth

INDIVIDUAL	NUMBER OF STRAINS*	AVERAGE INOCULUM	GAS IN CRYSTAL VIOLET BROTH			
			24 hours		48 hours	
			Number of strains negative	Number of strains positive	Number of strains negative	Number of strains positive
1	10	21	0	10	0	10
1	10	9	9	1	0	10
2	10	11	6	4	5	5
3	10	14	10	0	6	4
4	9	14	9	0	9	0
5	10	6	10	0	3	7
6	2	8	2	0	1	1
7	4	9	4	0	0	4
8	6	12	6	0	4	2
9	3	7	2	1	2	1
10	7	7	7	0	3	4
11	7	15	7	0	7	0
12	7	12	7	0	5	2
13	7	8	6	1	4	3
14	7	10	7	0	7	0
15	6	15	3	3	2	4
16	5	16	5	0	2	3
17	7	9	7	0	6	1
18	7	10	7	0	7	0
19	6	6	6	0	6	0
20	6	6	6	0	2	4
21	6	5	4	2	3	3
22	7	18	7	0	2	5
23	4	13	0	4	0	4
24	6	17	3	3	3	3
25	6	8	6	0	4	2
26	6	10	4	2	4	2
27	5	12	5	0	2	3
28	7	15	6	1	4	3
29	4	10	4	0	0	4
30	4	14	3	1	0	4
Totals....	201		168	33	103	98

* All ten strains from the first sample of Individual 1 were Aerobacter strains, two of the three strains from Individual 9 were also Aerobacter, all six strains from Individual 19 were Citrobacter strains. All other strains were typical Escherichia strains.

strains from 5 other individuals failed to form gas in the same period of incubation.

GAS PRODUCTION IN FUCHSIN BROTH

So few of the strains formed gas in fuchsin broth with the small inocula used in the crystal violet tubes that this part of the experiment was discontinued after 80 strains had been tested with small inocula. Of these 80 strains, only 5 produced any gas in the fuchsin broth in forty-eight hours. Later, all strains except the 5 which did produce gas from small inocula were tested in the fuchsin broth by adding 10,000 to 100,000 organisms. Out of all 201 strains tested by large and small inocula, only 33 or about 17 per cent produced gas in fuchsin broth, and 12 of these strains were from two specimens from the same individual.

EFFECT OF SIZE OF INOCULUM IN CRYSTAL-VIOLET BROTH

Ten strains, found to be quite sensitive to crystal-violet, were inoculated into the violet broth using different numbers of organisms in the inoculum. Of these 10 strains, 8 formed no gas in forty-eight hours with inocula as large as 1000 organisms per tube and in the case of 2 of these strains, an inoculum of approximately 1000 failed to produce any apparent growth within an incubation period of five days at 37°C. When the size of the inoculum was increased, all of these strains gave positive gas tests within forty-eight hours of incubation.

Of the strains which were found to be not sensitive to crystal violet in the concentration used, as few as one to three organisms were sufficient to produce gas within two days.

BACTERIAL SYNERGISM IN CRYSTAL-VIOLET BROTH

It has been known for some time that different pairs of organisms may produce gas in lactose broth while neither alone is able to do so. Sears and Putman (1923) found a number of such pairs of organisms which were able to give gas from lactose, sucrose or mannitol when neither would do so alone. Holman and Meekison (1926) found that in similar cases of bacterial synergism

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5	10	6	10	0	3	7
6	2	8	2	0	1	1
7	4	9	4	0	0	4
8	6	12	6	0	4	2
9	3	7	2	1	2	1
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11	7	15	7	0	7	0
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13	7	8	6	1	4	3
14	7	10	7	0	7	0
15	6	15	3	3	2	4
16	5	16	5	0	2	3
17	7	9	7	0	6	1
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19	6	6	6	0	6	0
20	6	6	6	0	2	4
21	6	5	4	2	3	3
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one organism of the pair must be capable of splitting the test substance, forming acid, while the other must be capable of forming gas from monosaccharides. Having noticed that gas production was apparently more likely to occur in a crystal-violet presumptive test fermentation tube when using a mixed culture found in a surface water, than when using pure cultures, there seemed to be a possibility of a somewhat different type of bacterial synergism than the ones to which reference has just been made. It was noticed when one-tenth of a milliliter of a heavily polluted surface water was added to crystal-violet broth, that not only was gas formed rapidly, but that the color of the dye was almost destroyed within twenty-four hours. An organism that would grow readily in the crystal violet and destroy the color was quite readily isolated and found to be a Gram-negative rod producing a greenish fluorescence on agar slants and abundant greenish pigment when grown in Sullivan's medium (1905). This culture was designated merely as culture "A."

When a loop of a broth culture of this green fluorescent organism was added to a tube of crystal violet, together with a small inoculum of one of the dye-sensitive strains of *Es. coli*, it was found that the fluorescent organism so altered the medium that the small inoculum of the dye-sensitive strain was able to produce abundant gas in an incubation period of thirty to forty hours while a similar inoculum alone in crystal violet did not show even a visible turbidity within forty-eight hours. Culture "A" alone produced no gas from lactose either in plain lactose broth or in crystal-violet broth in both of which media it grew readily and rapidly.

Several tubes of crystal-violet broth were inoculated with a loop of culture "A" and incubated sixteen to eighteen hours. A test made on two of these tubes showed no production of acid, but the purple color had almost disappeared. The remainder of these cultures of "A" in crystal-violet broth were heated at 100 to 103°C. for ten minutes and after cooling were inoculated with one of the most sensitive strains of *Es. coli*, culture 123, in decreasing amounts. The tube receiving approximately 10 organisms and all tubes receiving larger inocula produced abundant

gas within twenty-four hours of incubation, while the set of control tubes of crystal violet inoculated with similar numbers of organisms showed no gas with 1000 organisms even after an incubation period of four days. This experiment is summarized in table 2.

In order to find out whether a small inoculum of a dye-sensitive strain like culture 123 was killed by the dye, or was merely inhibited, a series of tubes of crystal violet were inoculated with decreasing numbers of these organisms. These cultures were placed in the incubator for twelve hours, at the end of which time each tube was inoculated with a loop of a broth culture of "A." After four days further incubation no gas was found in any tube that received an inoculum of 1000 organisms or less, indicating

TABLE 2

Effect of bacterial synergism on gas formation in crystal violet broth

APPROXIMATE INOCULUM OF CULTURE 123 OF BS. COLI	GAS IN CRYSTAL VIOLET IN 48 HOURS	GAS IN CRYSTAL VIOLET PLUS LOOP OF "A" IN 48 HOURS	GAS IN KILLED CULTURE OF "A" IN CYSTAL VIOLET 48 HOURS
10,000	Plus	Plus	Plus
1,000	None	Plus	Plus
100	None	Plus	Plus
10	None	None	Plus

that the organisms in the smaller inocula were all dead before the fluorescent organism had so modified the medium that they might start to grow.

It has been suggested by Ingraham and Fred (1933) and by Ingraham (1933) that the bacteriostatic effect of gentian violet (crystal violet used in the experimentation) is due largely to the poising effect of the dye on the oxidation-reduction potential. It was found that the growth of fluorescent culture "A" did reduce the oxidation potential. However, when the pH of the crystal violet was changed from about 7.4 to 6.0 by the addition of sulphuric acid and the oxidation potential was definitely raised, the dye had practically no inhibitory effect on dye-sensitive strains. On the other hand the addition of $CaCO_3$ to the crystal violet broth to change its pH to about 8.0 lowered the potential

but made the medium much more inhibitory to dye-sensitive strains of *Es. coli*. Just how the growth of culture "A" makes possible the rapid growth and gas production of dye-sensitive strains is uncertain. However similar synergism no doubt plays a considerable part in the production of gas by coli-aerogenes strains in water when added to crystal-violet broth. Hence crystal-violet broth would be expected to show more positive tests from polluted waters than would be expected from its poor showing in studying pure culture strains.

PRESUMPTIVE TESTS ON UNTREATED SURFACE WATERS

Twelve samples of different waters were tested for presence or absence of coli-group organisms by using plain lactose broth, brilliant-green lactose peptone bile, crystal-violet broth, and fuchsin broth. In 2 samples, plain lactose broth showed gas in higher dilution than did brilliant-green bile, and these positives were fully confirmed according to Standard Methods. On the other hand brilliant-green bile gave a higher index for coli-group organisms in 8 samples, 7 of which were confirmed. In the other 2 samples plain lactose broth and brilliant-green bile gave a similar index. The 2 samples which showed superior results for lactose broth gave positive tests in secondary tubes of brilliant-green bile.

Crystal-violet broth gave a coli index equal to plain lactose broth for 10 of the 12 samples of waters and gave superior results in the other two, both of which were confirmed. Fuchsin broth showed results equal to plain lactose broth in only 5 samples and inferior results in 7 samples.

DISCUSSION

Winslow (1934) has suggested that parallel planting in plain lactose broth and brilliant-green lactose peptone bile followed by secondary tubes of brilliant-green bile made from those tubes of plain lactose broth which do not also show gas in the brilliant-green bile primary tubes, be made the standard test on all waters without any attempt at further confirmation. The results of our studies both with pure cultures and the very limited number

of tests made on surface waters are in complete accord with the above suggestion of Winslow. Every one of the pure culture strains of Escherichia, Aerobacter, and Citrobacter grew almost equally well in brilliant-green lactose peptone bile and in plain lactose broth. On the other hand while crystal violet will no doubt give better results in mixed cultures as found in water samples than might be expected from its poor showing in pure culture studies, its use in presumptive tests is not advisable since quite frequently dye-sensitive strains would be likely to escape detection.

The poor showing of fuchsin broth would make its use inadvisable unless it can be shown that other samples of basic fuchsin than the one used (Coleman and Bell Certified No. CF-13) are much less inhibitory to coli-aerogenes strains.

SUMMARY

1. Pure culture strains of Escherichia, Aerobacter, and Citrobacter from 30 individuals, 201 strains in all, have been tested for gas formation in plain lactose broth, brilliant-green lactose peptone bile, crystal-violet buffered broth, and fuchsin broth.

2. When small inocula were used (less than 50 organisms per tube) lactose broth and brilliant-green lactose peptone bile gave positive tests after twenty-four hours of incubation at 37°C. for all 201 strains tested.

3. In crystal-violet broth after forty-eight hours of incubation using similar small inocula, only 49 per cent of these strains gave positive tests. From only 5 persons of the 30 from whom specimens were obtained did all strains produce as much as 10 per cent of gas in forty-eight hours, and all strains from 5 other individuals failed to form gas in the same period of incubation.

4. Very few strains formed gas in fuchsin broth when small inocula were used, and with relatively large inocula only 33 of the 201 strains were able to form gas in forty-eight hours.

5. When small inocula of a dye-sensitive strain of Escherichia were added to the crystal-violet broth and the tubes inoculated also with a loop of a culture of a greenish fluorescent water organism, the fluorescent bacterium so modified the medium that this

strain was able to produce gas in forty-eight hours or less. Hence less failures to get gas formation in the presence of Escherichia-Aerobacter organisms in water samples are to be expected than the poor showing of this medium when tested by pure culture strains would indicate.

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STUDIES ON ANAEROBIC BACTERIA

III. HISTORICAL REVIEW AND TECHNIQUE OF CULTURE OF CERTAIN THERMOPHILIC ANAEROBES

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INTRODUCTION

Although three groups of heat-resistant thermophilic bacteria have been shown to be important in the spoilage of non-acid canned foods, only two have been extensively studied from the standpoint of physiology and morphology. The group of thermophilic anaerobes, the suggested causal agents of the type of spoilage known as "hard swell," has received little attention in this respect. A cultural test (Bigelow, 1931) has been devised for the numerical estimation of these organisms, an invention of necessity in the solution of the practical problems arising from contamination of sugars, etc., but difficulties in cultivation have prevented detailed studies of the taxonomic characteristics of the cultures in question. Paine (1931) attempted such a study of a few cultures probably of this group, though purity of culture was not well established and certain of the results presented were incomplete, due to problems encountered in routine study. The investigation now reported presents a technique of culture and other methods essential to the preliminary studies and to the taxonomic and biochemical data which will be presented in later papers.

LITERATURE OF THERMOPHILIC SPOILAGE BACTERIA

Although, as early as 1895, Russell isolated bacteria from canned foods and later others (MacPhail, 1897; Prescott and Underwood, 1897; Poincarré and Macé, 1899; Harding and

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Nicholson, 1904, and others) also incriminated bacteria as a cause of spoilage of canned foods, it was not until 1912, with the pioneer work of Barlow, that thermophilic spoilage organisms received notice. The original report of Barlow is reviewed at length by Tanner (1924). More recently the problems of isolation and classification of some of these forms, together with studies on practical tests for their detection, have been investigated. In the last five to eight years a long list of publications has appeared on these topics, and because these are so widely scattered, a review of the literature is felt desirable.

There have been established three major types of thermophilic spoilage organis ıs in non- or semi-acid canned foods, the distinguishing characteristics of which have been reported by Cameron, Williams and Thompson, 1928; Cameron and Williams, 1928a; Cameron and Loomis, 1930; and Bigelow and Cameron, 1932. These three groups produce spores which possess unusual resistance to heat. They vary, however, in other respects.

The groups which have been recognized are as follows:[1]

1. *Flat sour organisms.* These are facultative aerobic bacilli, characterized by the production of acids (lactic, Bigelow and Cameron, 1932; and formic and acetic, Williams, 1934) but not of gas from carbohydrates. Obligate and facultative thermophilic sub-groups were found by Cameron and Esty (1926), who studied the cultural characteristics of 214 strains of this group. The type species is *B. stearothermophilus* of Donk (1920). Other descriptions of species similar or related to the "flat sour" organisms may be found (Wyant and Tweed, 1923; Bitting, 1924; and Shaw, 1928).

[1] The grouping presented is that commonly used in the literature of this subject. It is recognized that, as the non-toxic food spoilage organisms are more extensively studied, it will be necessary to extend or change any system of grouping which may be proposed at the present time. It may, perhaps, be preferable to classify the various groups according to the outward appearance of the can of spoiled food after inoculation, followed by suitable incubation, from a pure culture of the organism in question. Such a classification would provide for two main divisions; those in which the can has been swelled and those in which no outward evidence of the spoilage is observed. In this case the "flat sour" (producing no gas) and the "sulfur stinker" (producing a soluble gas) groups would appear as sub-groups of the latter division.

2. *Gas-forming (non H_2S) anaerobes.* These are the so-called "thermophilic anaerobes" or "T. A." cultures with which this study will be chiefly concerned. By production of gases[2] (carbon dioxide and hydrogen) in large quantities from carbohydrates these forms are responsible for the spoilage known as "hard swell," in which the can may be bulged or the seams split due to the pressure; a "cheesy" odor is sometimes noticed in certain spoiled canned goods or in cultures of this type. Other than the reports of sources from which these forms may be isolated the only published study of this group is that of Paine (1931).

3. *Sulfid or "sulfur stinker" organisms.* The distinctive feature of this group is the production of hydrogen sulfid at thermophilic temperatures. The causal organism was originally classified by Werkman and Weaver (1927) and named by them *Clostridium nigrificans.* Werkman (1929) reported a detailed study of the cultural characteristics. *Clostridium nigrificans* is strictly non-saccharolytic and feebly proteolytic, attacking cystine with the production of hydrogen sulfid; thermophilic temperatures are optimum for growth. Although spoilage due to this species has not been common, an outbreak is serious (Cameron, 1928).

Efforts have been made to discover the source of the spores of thermophilic spoilage forms in nature. Although the ultimate source may be the soil, practical problems in the canning industry have caused more immediate sources to be examined. Sugar and starches have been found to be important sources of these organisms in canneries (Cameron and Williams, 1928a and 1928b; Cameron and Yesair, 1931; Owen, 1932; Owen and Mobley, 1932; and Clark, 1933).

Other reports have shown the widespread contamination of sugars by thermophilic spore-forming bacteria of some one of the above mentioned classes (Cameron, 1928; James, 1928a, 1928b and 1928c; Williams, 1930; Cameron and Bigelow, 1931; Barnes, 1932; Owen and Mobley, 1933; and Clark, 1933). Recognition

[2] According to the figures of Paine, the carbon dioxide-hydrogen ratio in the fermentation of glucose is about 1:8, although the time at which the analyses were made was not given.

of this fact led to demands for uncontaminated sugars by the canneries. Tentative standards for thermophilic purity of sugar were suggested in 1931 by the National Canners' Laboratory (Bigelow, 1931). Sugar that is "certified" to meet these standards is now available. Contamination from infected bagging has also been considered (Owen and Mobley, 1932; Owen, 1932).

In addition to the contamination of particular lots of foods a sugar containing these organisms may present a greater danger because of the contamination of the plant itself as, for example, the establishment of the organisms in wooden brine lines and tanks (Cameron, Williams and Thompson, 1927 and 1928; and Cameron and Bigelow, 1931). Such equipment may serve as a constant source of contamination for all products canned, and, since the spores of these bacteria survive a more severe heat treatment than the maximum possible without injury to the food materials (Cameron, 1931; Cameron and Yesair, 1931a, 1931b; and Townsend, 1932), the spoilage losses may be great (Bigelow and Cameron, 1932).

That the raw product at times may be sufficiently contaminated to induce spoilage has been reported by Townsend (1932) in the case of asparagus. The source of contamination here was the soil and other reports have also shown the spoilage forms to be present in soil, usually that which is heavily fertilized (Werkman and Weaver, 1927; James, 1928b and 1928c; Cameron and Williams, 1928b; Werkman, 1929; Medaris, 1931; and Clark, 1933).

The possibility of economic loss due to these forms is not confined to this country. Prickett (1932) detected thermophilic spoilage bacteria in canned Italian shallots. The presence of the recognized groups in Belgian, Russian, Czechoslovakian and Dutch sugars was established by Homans (1933a and 1933b). Although Hirst (1931–2) reported isolated cases of thermophilic spoilage in England, no serious outbreak had occurred. He suggested that the low temperature of storage in English warehouses served to minimize the danger.

Occasional reports, *e.g.*, Duval (1931) may be found in which the author does not agree with these generally accepted opinions

as regards thermophilic spoilage bacteria. In these reports the absence of experimental procedures and of the exact results eliminates from serious consideration the criticisms offered. The validity of the conclusion that sugar may play an important rôle in the contamination of a plant has been questioned by Ingersoll (1930a and 1930b), who believes thermophilic contamination to be a matter of sanitary condition of the plant. This view, however, is not general (Bigelow, 1930), and specific answers to Ingersoll's criticism have been given (Cameron, 1930b and Williams, 1930).

Many report the presence of viable bacteria in supposedly sound canned foods. The pioneer work on this problem was done by Cheyney (1919), Weinzirl (1919) and Savage (1923). Occasional thermophilic types were encountered but descriptions of the cultures are so incomplete as to make impossible accurate identification of the species involved. Hanzawa and Takeda (1931), in a study of the bacteriology of "swelled" cans of cod-fish-cake, include a review of some of this literature.

MEDIA USED AND TECHNIQUE OF CULTURE

At the outset of this study of the thermophilic gas-producing (non-H_2S) anaerobes, it seemed evident from the literature and communications with those studying the practical side of spoilage problems, that special procedures would be necessary if consistent results were to be obtained. As the work progressed this prospect became a reality. As will be pointed out in the appropriate sections, many of the procedures which have been found successful for other anaerobes were not satisfactory for these thermophilic anaerobes. Two major problems which presented unusual difficulty concerned growth on the surface of solid media and the controlled production of spores in laboratory media.

Media

Difficulty was experienced in choosing a medium for general cultivation purposes. In a study of the enrichment media suitable for this group, Medaris (1931) was unable to find many that would be useful. In addition to the liver infusion of Cameron, Williams and Thompson (1928), potato glucose infusion and

certain of the infusions prepared from canned strained vegetables were sometimes satisfactory. An extension of these experiments has been necessary and the essential types of media tried, together with methods for their preparation, will be discussed.

Medium for enrichment and general cultivation purposes. Liver infusion (liquid infusion tubed and sterilized over particles of dried extracted tissue) has been the most extensively used for the group, as it seems peculiarly suited to their growth requirements. It is the medium recommended for commercial use in detecting contamination of sugars and other materials, but in the present study a slight modification of the original formula has been used (McClung and McCoy, 1934). Growth is indicated by turbidity of the infusion above the particles of ground tissue and a violent production of gas during the early stages of growth; the liquid also becomes lighter in color. The odor is distinctive but not that of putrefaction.

The infusion as usually prepared is probably many times more concentrated than is necessary; a 1:10 dilution allows growth, although if dilute inocula are used the time for the appearance of turbidity and gassing is lengthened. It is important that previously sterilized tubes be used for this medium, as the time of autoclaving should not exceed forty to forty-five minutes (17 pounds pressure). Further sterilization darkens the medium and renders it unsuitable for growth. The danger of over-sterilization of liver medium has also been observed by Jackson and Muer (1911).

This medium, although very satisfactory for cultural purposes, is not to be recommended for material for stained mounts. Certain Gram-positive, granular organisms, which apparently multiply rapidly on the liver tissue during the drying process, appear in certain batches. These forms are, however, characteristic and may be recognized and disregarded.

Other media commonly used for other types of anaerobes were found unsuitable for various reasons. The gassing and turbidity diagnostic of growth of the thermophilic anaerobes in Robertson's (1931) alkaline meat medium, Hall's (1920) brain medium and others of similar type are transient, thus making the indications

of growth uncertain. Difficulty of preparation and sterilization of these media also make them undesirable for general routine purposes. Beef-extract peptone broth and the various peptone solutions tested are not suitable, nor is yeast water, even with the addition of 1 per cent glucose as an additional carbon source and reducing substance, satisfactory. The poorly buffered excelsior extract medium proposed by Paine (1931) for this group did not supply the essentials for growth; addition of glucose or microcosmic salt as suggested did not improve the medium. As has been previously noted (McClung and McCoy, 1934), plain cornmeal mash is not good for this group.

Plating medium. As was earlier demonstrated by Medaris (1931) certain of the vegetable media have proven useful for tube culture. Five to 10 per cent infusions of canned strained vegetables (peas, spinach, etc.) are made, and for plating purposes, 2 per cent agar is added. The inability of certain of these vegetable infusions to support growth is probably due to the extreme pH change which occurs during sterilization, as has been previously suggested (McClung, 1932).

Pea infusion seems to undergo less change of reaction than others and will support consistent growth; it has been almost exclusively employed as a plating medium in this work and can not be recommended too strongly for this purpose in culturing this particular group. It is inexpensive and easy to prepare, fairly well standardized, and allows spore formation. This last point is particularly important as many of the other media which do allow growth on solid media as, for example, liver infusion or glucose agars are unsatisfactory in this respect.

Base medium for physiological studies. Some difficulty has been encountered in finding a suitable base medium for the study of the physiological characters of these cultures. Peptone solutions (proteose, bacto and Witte) in various concentrations, beef-extract peptone broth, yeast water and others tested are not to be used, for even with the addition of 1 per cent of carbohydrate inconsistent results are often obtained, almost uniformly so if dilute inocula are used. Casein digest, prepared according to the formula of Kristensen, Lester and Jürgens

(1925), is somewhat better in this respect but has the disadvantage of variation from batch to batch. The most satisfactory base which has been found is 1 per cent tryptone broth[3] (in distilled or tap water with no adjustment of pH) to which the various test substances are added. Little or no growth is obtained in the base alone; glucose in 0.1 to 1.0 per cent concentrations stimulates growth, the time required for the development of turbidity of the medium or for gas production as indicators of growth being inversely proportional to the amount of sugar added.

Other media. A new medium was devised for certain correlated problems requiring large numbers of tubes, for which none of the above described media was satisfactory. The details of preparation of this medium, containing 1 per cent dried ground liver tissue and 5 per cent cornmeal have been described in an earlier publication (McClung and McCoy, 1934) and need not be repeated. The results which have been obtained indicate that the medium is satisfactory for routine cultivation purposes, *i.e.*, serial transplant studies, sub-culture following thermal death time tests, examination of sugars, starches, soil, etc.

Other media have been prepared according to standard procedures or specific reference will be given as needed. Unless otherwise noted all media have been sterilized by autoclaving for thirty minutes at 17 pounds pressure. The usual practice of exhaustion by steaming of the tubed media before use for anaerobic culture, has been followed except in the case of the corn-liver medium, for which this does not seem necessary.

Technique of culture

Test tube. It has been commonly noted that consistent positive results with this group will not be obtained in tube culture if the medium is not protected from the air. This appears to be true even with the more reduced media like liver infusion or glucose broth; the use of large inocula in part avoids this difficulty but is often impossible or undesirable. This fact has been met

[3] This medium is prepared from the desiccated product "Tryptone" of the Difco Laboratories; formerly sold as "Tryptophane Broth."

by others working with anaerobes and the devices to circumvent the problem have been many. Various mechanical seals, *i.e.*, vaseline, oil, paraffin, etc., have been proposed but the inconvenience of these procedures in routine culture scarcely warrants their use. The incubation of all tubes in anaerobic jars offers another solution but one which can not be applied if the tubes require inspection at close intervals, nor is it convenient to use many of the available anaerobic systems at thermophilic temperatures.

The addition of inert substances to certain media for promoting anaerobic growth is an old method which many have found useful. These substances are of several types and a partial review of their uses may be found in the recent publication of Breden and Buswell (1933). Several of these—chalk, asbestos, cotton, glass wool, pumice stone, small percentages of agar, etc., —were tested in various base media but without success in the present work. The use of particles of dried liver tissue was advantageous with certain bases but the action here is probably due as much to the soluble nutrients from the liver as to the reducing action of the tissue.

Cameron, Williams and Thompson (1928) recommend in enrichment cultivation of this group the stratification of liver infusion with yeast water agar made by pouring sterile agar into the inoculated tube, thus giving a series of physical phases—from liquid to jel—in which growth occurs. Medaris (1931) modified this method by first adding to the surface of the inoculated tube a thin layer ($\frac{1}{4}$ inch) of sterile paraffin, which was allowed to solidify; then sterile non-nutrient (2 per cent) agar was added to form a 1 inch seal. This seal is effective but the paraffin feature complicates washing of tubes, a factor to be considered if many tubes are involved.

In some cases, as liver infusion, the paraffin layer is unnecessary if the agar is added carefully. The plain agar seal is also possible on most other media (vegetable infusions, milk, tryptone broth, etc.) but only if the following precautions are observed. Cool the inoculated tubes by immersion for several minutes in ice water and add two or three drops of 3 per cent agar to form a

thin layer on the surface of the medium. After this has solidified (two or three minutes) add the remainder of the agar as before. Contamination of tubes prepared by this method is rare and may be reduced to a negligible amount by the addition of gentian violet (1:50,000) to the agar used for sealing. The disadvantage of the extra time required to prepare tubes by this method is offset by the assets which it presents—ease of examination for hourly or daily readings; ease of removing inoculum or material for staining; elimination of the need for anaerobic jars adapted to thermophilic temperatures. This modification of the original method of Cameron and Williams has been used throughout our study.

In the use of the agar sealed media it has been found useful to transfer with capillary pipettes drawn from 7 mm. (inside diameter) glass tubing in 10 cm. lengths which are thrust between the agar and the side of the tube in obtaining culture material. One or 2 ml. rubber bulbs are used for drawing up and expelling. The pipettes may be cleaned and used repeatedly.

Occasionally it is desired to incubate cultures not under seal for periods of forty-eight to sixty hours in which case evaporation at the thermophilic temperatures becomes a serious factor. If the medium is semi-solid or of such nature that protection from oxygen is not necessary for anaerobic growth, wrapping of each tube in lead foil will suffice to delay evaporation. If much longer incubation is required, all tubes may be enclosed in a jar.

Plate culture technique. Following the development of technique which made possible consistent results with various tubed media, it seemed logical that growth on the surface of solid media would be obtained if the same base media and inocula were used and anaerobic incubation was provided. This, however, was not found true. In an attempt to have as few variable factors as possible, one standard source of inoculum was chosen for the early experiments; this was an eighteen to twenty-four-hour culture of the strain in question in liver-infusion broth under agar seal. Proposed devices for anaerobic plate culture are so numerous in the literature and the more practical ones are so widely known that specific references are unnecessary. It is

enough to state that a large number of these were tested. Following preliminary experiments the Novy jar (replacement with carbon dioxide, nitrogen, or a mixture of carbon dioxide and hydrogen), the phosphorous jar of Sellards (1904) and the vegetable tissue system of Carroll and Hastings (1925) were chosen for detailed study. In these were tested all the media which were known to be favorable to growth in tube culture; in each case the standard inoculum (active liver culture) was used. The results were almost uniformly negative. Any positive culture seemed a matter of chance for it could not be duplicated using, as nearly as possible, identical conditions.

It was later shown that the inoculum was the factor responsible for these negative results. In one experiment the use of aged cultures in liver or vegetable infusion as the inoculum for plate culture resulted in colony formation. Subsequent experiments showed that colony formation could be induced in every culture by this method; furthermore, if the plating medium used were a vegetable-infusion agar (strained peas), spores would be produced in the colonies formed providing an incubation time of four to seven days were allowed. The use of old cultures or previously prepared spore suspensions is now considered to be an absolute essential for successful plate culture regardless of the type of anaerobic system used. Positive cultures have thus been obtained in anaerobic environments provided by various methods whereas completely negative results have resulted with every plate culture incubated in the same jars in which the inoculum was from a young culture. No difficulty in plate culture has been encountered in any instance since this method was adopted as a routine procedure; while the complete reverse is true in any case where this is not observed.

The vegetable tissue system (potato) of Carroll and Hastings (1925), which has recently been shown to be effective at thermophilic temperatures (McClung, McCoy and Fred, 193–), has been used in routine culture with complete success. With this method it is necessary to use clay tops on the petri dishes to absorb the excess moisture formed. The use of these clay top dishes does not over-dehydrate the agar, as might be expected

at the high temperature of incubation, leaving a surface that is too dry for proper growth but on the contrary the dryness aids in the formation of discrete colonies, a distinct advantage in the purification of cultures or in the study of colony types. This dry surface also seems to be an essential in the production of a high yield of spores. At least 20 ml. of agar should be used in each dish; a smaller amount is not to be recommended since a minimum of forty-eight hours of incubation is necessary for the proper growth of colonies and ninety-six to one hundred twenty hours for sporulation.

Spore formation. The best method which has been found for the production of spores is the growth on the surface of suitable agar, using the petri dish technique described above. The vegetable-infusion agars (usually of strained peas) are to be used since liver, glucose tryptone or similar agars do not allow spore formation. If it is desired to build up a stock spore suspension, it is best to develop the culture by serial plating technique rather than by using tube cultures for inoculum. Dilutions of the growth may be prepared in tap water or physiological saline and these serve as a stock inoculum for a number of plates; these stock spore suspensions remain viable for considerable periods of time. For the production of spores the petri plate technique is preferable to growth in bottles, as the plate culture gives a better yield of spores even in the same medium; it is probable that moisture relations explain the difference.

Stock cultures. In any problem of this nature it is essential to have on hand stocks from which physiologically comparable cultures may be obtained. In the culture media which permit the most luxuriant vegetative growth (liver infusion, etc.), it will be found that after a certain period of incubation the cultures of this group will become autosterilized or nearly so, and for this reason such media are not to be recommended for the maintenance of stock cultures. It has been previously mentioned that stock suspensions of spores in tap water (or physiological saline solution) remain viable apparently indefinitely for although exact counts were not made, dilute water suspensions of spores (in bottles closed by metal caps) were viable after eight months

of storage at room temperature. Another method of preserving the stock cultures of this group is to dry spore suspensions in tubes of sterilized garden soil. Soil stocks prepared in this manner were viable after a period of several months with no indication of loss in the numbers of viable spores. Similarly, liver cultures dried upon particles of liver may serve as reference culture sets. Although it has never been possible to demonstrate the presence of spores in liver infusion cultures by microscopic examination, a very low percentage of spores is probably formed.

SUMMARY

Three major groups of thermophilic bacteria have been established as causal organisms in certain types of spoilage of non-acid canned foods. Of these one group, the thermophilic gas-producing (non-H_2S) anaerobes, has not previously been extensively examined and this group has been chosen for study. Methods are presented which are believed to be satisfactory for use in routine study. These include the formulae of various types of media which have been found suitable for growth as well as especial technique necessary in test tube and plate culture. A method which has been found to be satisfactory for the controlled production of spores in the laboratory, by surface growth of the cultures on vegetable extract agar is discussed. Methods for the maintenance of stock cultures are included.

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STUDIES ON ANAEROBIC BACTERIA

IV. TAXONOMY OF CULTURES OF A THERMOPHILIC SPECIES CAUSING "SWELLS" OF CANNED FOODS

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HISTORY OF STRAINS

In a previous paper (McClung, 1935), methods of culture were discussed for the isolation and routine cultivation of a group of thermophilic anaerobic spore-forming bacteria. It seemed necessary to study the technique of culture of this particular group in some detail as in many instances it was found that either media or methods which have been used by other investigators for the various groups of mesophilic anaerobes were not satisfactory for the routine study of this group. Following the development of these methods a detailed study has been made to aid in the classification of the cultures. As was earlier mentioned in the review of the literature, little has been published on the morphological and physiological characteristics of this group.

Sources of strains

In an attempt to obtain as nearly as possible a representative collection of cultures, strains were secured from the National Canners' Research Laboratories in Washington[1] and original isolations were made from soil.[2] Seven cultures were obtained from the Washington laboratory; twenty represent original isolations. Included in the first seven are strains which had been isolated from "blown" canned foods or from sugar. The soil dilutions

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[1] Through the courtesy of Dr. E. J. Cameron.

[2] The soil used was obtained from Dr. J. R. Esty of the Western Branch of the National Canners' Association. The samples were from the contaminated field reported by Townsend (1932).

(1:100 and 1:1000) were steamed for fifteen minutes (thus eliminating almost entirely the usual contaminants) before subcultures were made into liver infusion with 1:100,000 gentian violet (as recommended by Cameron (1930) to reduce the "flat sour" contamination) and into pea infusion which were incubated at 58°C. for enrichment.

Purification of cultures

Following several serial transplants in the enrichment media, plate cultures on pea-infusion agar were made. Serial (six or more) single colony isolations by the streak technique served as the purification procedure. Microscopic examination followed each step of the purification; in no case did forms other than the typical one (to be described later) appear after the plating began. The cultures received from the Washington laboratory were subjected to the same re-purification procedure. Single cell isolation[3] was used as the final step in the purification of six of the cultures. All cultures were then tested for both aerobic and anaerobic contaminants at 37°C. and 55°C. and for their ability to produce the "hard swell" spoilage at thermophilic temperatures in cans of peas or corn which had been previously incubated at 60°C. to insure their freedom from this group of organisms.

DISTINCTIVE TAXONOMIC CHARACTERISTICS

Morphological characteristics

Morphology. All the strains are long, slender, granulated bacilli bearing a round, strictly terminal spore which swells the rod (figs. 4 and 5). The organisms occur singly or occasionally in pairs, though never in chains. Although there is some variation in the size of the individual cells within the same culture in a

[3] *Method.* Spore suspensions were obtained from surface growth on vegetable infusion agar and single spores isolated by means of a modified Chamber's micromanipulator. The tip of the micropipette containing the isolated spore was clipped with sterile forceps and dropped into tubes of liver infusion or glucose tryptone broth (agar seal). Although only a small number of spores was isolated, a fair percentage of these germinated. Isolation of vegetative cells was not attempted as this has been shown by others to yield a lower percentage of positive cultures with other groups of anaerobic bacilli.

particular medium, the general picture is characteristic; a change of the culture medium, however, alters the cell type. In the glucose-containing media (glucose tryptone broth, etc.) the vegetative cells become shorter and thicker than those in vegetable-infusion or liver-infusion media; also no sporulation is seen. Cell measurements of a four-day-old culture on pea-infusion agar (air-dried nigrosin mount) showed the following variations: length, 3.5 to 7.5μ and width, 0.4 to 0.7μ. Spores from the same culture varied from 0.6 to 0.8μ in diameter. These are similar to the figures reported by Paine (1931).

The mode of sporulation is the most distinctive feature of the microscopic picture. On pea-infusion agar young spores may be seen as early as forty-eight hours; ninety-six to one hundred and twenty hours incubation is usually necessary for these to mature. Free spores are not often observed; the sporangium remains attached to the spore even in cultures in which spores which take a spore stain have been observed for several days.

Staining reactions. The vegetative cells of these cultures are often granulated; this is especially well revealed by methylene blue or dilute gentian violet. Granulation is most pronounced in old cultures in the process of spore formation or in cultures grown in media which are not well suited for growth. Immature spores stain more intensely with the simple stains than do the sporangia in which they are borne. Mature spores are stained only with difficulty; the methods of Dorner and of Moeller and the technique recently proposed by Schaeffer and Fulton (1933) have all been used with success. Irrespective of the method used, the heating process in the staining of the spore should be lengthened from five to ten times that proposed for the mesophilic aerobic spore-forming group.

None of the cultures of this group are stained by Gram's method. This appears to be an exception to the rule that spore-forming organisms are Gram-positive (although other exceptions may be found in the literature, *e.g.*, aerobic spore-forming bacilli causing false presumptive tests in water analyses) and is also contrary to the report by Paine (1931). Although in this paper the vegetative cells are reported to be easily decolorized the technique of

the stain was not included and thus the exact procedure can not be checked. In addition to the Gram stains made in the usual routine of study, the following experiment was carried out to test this point. Five strains were grown in liver infusion and smears prepared on acid-cleaned slides at the end of three, six, nine, twelve, fifteen, eighteen and twenty-four hours following inoculation. Quadruplicate slides were prepared and the smears of two sets received, before drying, a dilute suspension of *Lactobacillus Delbrückii*. One set of the slides prepared in this manner and one set without the added organism were stained by the Kopeloff-Beerman modification of the Gram stain (acetone-alcohol used as the decolorizing agent); the remaining two sets were stained by the Hucker modification (95 per cent alcohol as the decolorizing agent). In no case were the cells of the thermophilic species Gram-positive, though the lactobacillus did take the stain. In a few instances, not correlated with the age of the culture but probably with slight variation in decolorization, the cells of the thermophilic organism assumed a slight purple tinge; in no case were they definitely Gram-positive.

Upon examination by the Hiss method no capsule could be demonstrated. The granulose reaction with Meissner's iodine solution was negative in twenty-four-hour cultures grown in corn-liver medium.

Motility. A small percentage of the cells of a four- to six-hour-glucose tryptone broth were found to be slowly motile by microscopic examination. The inocula for this series were from active (eighteen-hour) cultures and the tubes were preheated to 60°C. before inoculation. Peritrichiate flagella are seen in stains prepared according to the methods of Gray (1926) and Craigie (1929). Older cultures (eighteen to twenty-four hours) which have been repeatedly examined by the coverslip method or, in certain instances, by the capillary tube technique of Sturges (1928) do not exhibit motility.

Colony characteristics. Deep colonies are rather difficult to obtain, for the best media for colony formation are those containing glucose. The concentration of sugar necessary for growth allows extensive gas production, resulting in bubbles and cracks

in the agar in the vicinity of the colonies. Deep colonies, in a glucose (0.5 per cent) tryptone (1 per cent) medium in which the agar concentration was reduced to 1.5 per cent, are small and lenticular.

The usual surface colony type on pea-infusion agar is of medium size (2 to 4 mm.), granular, with rough, indistinct, "feather" edges, greyish-white and often showing a slightly raised center, if the colonies are well isolated (fig. 1). On prolonged incubation (three to five weeks) at thermophilic temperatures, secondary colonies may sometimes be observed (fig. 3). Two cultures, as originally isolated, did not show the colony type described above but are, in general, smaller and without granulations, the edge being smooth (fig. 2). Recent studies have shown that the phenomenon of induced variation of bacterial cultures is not confined to the aerobic species but may be true also of the common anaerobic species. The colony types observed in this study may indicate such a phenomenon although it is not possible to state whether or not the two colony types represent true "rough" and "smooth" phases as this problem has not been extensively studied. The descriptions given are those shown by the cultures as originally isolated; they have remained stable during a period of eighteen months. The two types are not separated by the cultural reactions applied.

Physiological characteristics

Oxygen relations. All cultures are obligate anaerobes growing only in the more reduced media with strict exclusion of free oxygen. Even with media of low oxidation-reduction potential such as liver infusion with dried liver tissue, or others of similar nature, growth will not occur in unsealed tubes unless a large inoculum (1 to 2 ml.) of an active culture is used, and the medium must be previously exhausted of dissolved air by steaming. Their anaerobicity is also shown in agar shake cultures (0.1 per cent glucose, 1 per cent tryptone agar) in which there is an inhibition of growth for about ½ inch from the surface. The failure to obtain plate cultures with vegetative inocula, as previously discussed, is also evidence for considering the group strict anaerobes.

The limiting oxidation-reduction potential for growth has not been determined. The catalase reaction is negative.

Temperature relations. With the exception of the experiment next to be described cultures for all other tests have been incubated at 58° to 60°C. This is well within the optimum range for growth as is shown by the following series in which a study was made of the temperature relations. The temperatures chosen were 20°, 30°, 37°, 42°, 55°, 62° and 70°C. Both spore and vegetative inocula were used for all cultures. In the case of the vegetative cells the inoculum was a 1:100 dilution of an active liver-infusion culture; this had been shown in preliminary experiments to be sufficiently dilute so that the lag phase would be pronounced with unfavorable conditions. A similar dilute inoculum for the spores was prepared from a stock suspension which had been heated sufficiently to kill the vegetative cells. The medium was liver infusion, tubed and sterilized over particles of dried liver. Duplicate tubes from both spore and vegetative inocula were made for every culture at each temperature and, following inoculation, all tubes were sealed with a plug of agar.

A summary of all the results of this experiment may be seen in table 1; the abundance of growth, as indicated by the volume of gas produced and the turbidity of the infusion, is indicated numerically. The results were so nearly uniform that it is unnecessary to report the specific details for each culture. In general, the cultures at thermophilic temperatures (55°C. and 62°C.) from the spore inocula were identical with those from vegetative inocula. At lower temperatures, however, growth initiation from the spore inocula was a little slower than from the vegetative inocula. These results show that the optimum temperature is thermophilic; although, if prolonged periods of incubation are used, germination of spores and growth is possible at lower temperatures. The practical importance of this slow growth as a factor in spoilage losses in canned foods is obvious.

Heat resistance. The exact heat resistance of these cultures, has not yet been determined. That the resistance is high has been repeatedly shown in experimental packs of non-acid products by the control laboratories and by the fact that the spoilage

produced by these organisms in commercial canning is due to the survival of the process temperatures by the spores. Confirmatory evidence is found in the fact that the soil dilutions used for enrichment material in this study were subjected to steaming and also that all spore suspensions used as inocula in the experiments reported in this study were steamed from fifteen to twenty-five minutes.

TABLE 1

General results on temperature relations of cultures receiving vegetative inocula

TIME	TEMPERATURE OF INCUBATION							
	20°C.	30°C.	37°C.	42°C.	47°C.	55°C.	62°C.	70°C.
days								
1	0	0	0	0	1	4	4	0
2	0	0	0	0	2	—	—	0
5	0	0	0	2	3	—	—	0
10	0	0	1	3	4	—	—	—
20	0	0	2	3	—	—	—	—
30	0	±1	2	—	—	—	—	—

"1, 2, 3, 4," symbols indicating progressive stages of growth; "0," no visible growth; "—," discarded.

Diagnostic cultural characteristics

Growth in complex media. The members of this group are not separated by the differential media usually considered in the classification of anaerobic bacteria. The general reactions produced in the various complex media or in certain differential media by all the cultures are given in table 2. Brain mash and beef-heart infusion, which have perhaps been most widely used for general cultivation of other groups of anaerobic bacteria are not suited to this group. Growth is obtained only from large or active inocula and the reactions produced are not easily recognized or particularly distinctive. A better medium for this group is liver-infusion broth sterilized over particles of dried liver as described in the previous paper. Gas and turbidity of the infusion are easily recognizable indicators of growth; the infusion liquid also becomes lighter in color.

Proteolytic properties. In brain mash, beef-heart infusion, etc.,

the cultures do not exhibit proteolytic powers; this confirms the conclusion of Paine (1931) which was based upon formol titrations. Further, they are not gelatinolytic as shown by the Frazier technique, using both tannic acid and mercuric chloride as precipitants, applied to five day old cultures in tryptone agar containing 0.1 per cent glucose to promote growth.

TABLE 2

Reactions following growth in complex media

MEDIUM	REACTION
Brain mash	Slight turbidity with transient gassing. No blackening, even on prolonged incubation. No proteolysis
Beef heart infusion	Slight turbidity. No digestion of the meat tissue or putrefactive odor
Liver infusion	Marked turbidity; violent gassing in early stages (12 to 20 hours) of growth. Liquid becomes lighter in color. Distinctive odor but no digestion of the tissue. Marked auto-agglutination; particularly if cultures are removed from thermophilic temperatures
Alkaline egg medium	Gassing (with addition of 0.5 per cent glucose) but no digestion of coagulated albumin. No visible growth in absence of sugar
Blood agar plates	Slight growth only. It is not possible to determine the type of hemolysis due to the temperature of incubation. (Horse blood; tryptone base)
Coagulated serum slants	Slight or no growth. No liquefaction
Corn-liver mash (5 per cent)	Gassing in early stages of growth. Slight clearing after 24 hours
Litmus milk (with reduced Fe)	Gas production in the early stages during which time the litmus is reduced. A hard curd is formed which may be channelled by subsequent gassing. The litmus may be re-oxidized on further incubation and tricalcium citrate crystals appear after 8 to 10 days (Peterson, *et al.*, 1927)

Saccharolytic properties. Growth does not occur in the absence of a fermentable carbohydrate, whereas the saccharolytic properties of the cultures are extensive and well defined. The qualitative fermentations of the various sugars and related compounds reveal an unusual uniformity of results with respect to the compounds fermented by the several strains, regardless of their separate origin.

The criteria to be used in anaerobic fermentation have been a matter of dispute (Hall, 1921) and probably any one method will not be acceptable to all. In this study two criteria have been considered. First, in the base medium (1 per cent tryptone broth) alone no growth or only very slight turbidity will occur (the inoculum used in this experiment was 1 ml. of a 1:100 dilution of active liver infusion culture). Thus growth in the presence of a carbon compound becomes an indicator of the utilization of the added compound. An increase in the hydrogen ion concentration becomes an additional criterion, since, as has been previously shown, the strains are non-proteolytic; and therefore, the acid can not result from protein cleavage. For the same reason, there has been no complication due to the production of ammonia which might unite with the acids produced. Therefore, in the present case, a change of pH may be taken as a useful index of fermentation. As will be discussed later, because of the uniformity of results and the exact correlation of positive results according to the two criteria used, it was not considered necessary to test for the absence of the compound utilized, in the case of those substances for which a practical method of quantitative determination exists.

The various carbon compounds were sterilized in concentrated aqueous solutions, and added to give a concentration of 1 per cent in the sterile base. Duplicate tubes, inoculated from a 1:100 dilution of an active liver-infusion culture, were incubated at 58°C. The unsealed tubes were incubated for five days in an anaerobic jar. The results are shown in table 3. The values in the final pH column represent not the range of duplicate tubes of any one culture, but the range of the entire set of cultures. Thus, for instance, the final values for all cultures with cellobiose fell within the range of pH 4.1 to 4.4; whereas with glucose, fructose, etc., no difference in the final pH reached by all the cultures could be detected by colorimetric methods. In no case was there a disagreement between growth and change in pH.

As shown in table 3, the strains tested ferment all the mono- and disaccharides (with the exception of the methyl-pentose, rhamnose) and also all the glucosides tested. The action on the trisaccharide, raffinose, is slight, if any; the alcohols tested and

calcium lactate are not utilized. Of the polysaccharides inulin and pectin are not fermented in contrast to dextrin, glycogen and

TABLE 3

Qualitative fermentation of sugars and related compounds

	GROWTH	FINAL pH	CONTROL
Monosaccharides:			
Arabinose	+	4.6–4.7	6.2
Fructose	+	4.3	6.3
Galactose	+	4.3	6.3
Glucose	+	4.3	6.3
Mannose	+	4.3	6.3
Rhamnose	−	6.2	6.2
Xylose	+	4.2–4.3	6.3
Disaccharides:			
Cellobiose	+	4.1–4.4	6.3
Lactose	+	4.5–4.7	6.3
Maltose	+	4.3	6.4
Sucrose	+	4.4–4.8	6.3
Trehalose	+	4.3–4.8	6.3
Trisaccharide:			
Raffinose	±	6.3	6.4
Polysaccharides:			
Dextrin	+	4.3–5.2	6.3
Glycogen	+	4.3	6.4
Inulin	−	6.3	6.3
Pectin	−	6.1	6.1
Starch (corn)	+	4.6–4.9	6.4
Glucosides:			
Amygdalin	+	4.3–4.6	6.6
Esculin	+	4.9–5.2	6.5
Alpha methyl glucoside	+	4.5–4.8	6.4
Salicin	+	4.3–4.9	6.3
Alcohols:			
Erythritol	−	6.4	6.4
Inositol	−	6.3	6.3
Mannitol	−	6.4	6.4
Glycerol	−	6.3	6.3
Quercitol	−	6.3	6.3
Salt of organic acid:			
Calcium lactate	−	6.4	6.4

corn starch. Only very slight growth is obtained on potato slants, a high starch-containing medium, and no distinctive picture is pre-

sented. Slight action on arrowroot starch is observed by the giant colony technique on agar. The discrepancy of results in the case of starch hydrolysis possibly correlates with the origin of the starch—whether from grain or tuber. The action on cellulose was determined by the addition of strips of filter paper to tryptone base medium, and to the same plus 0.1 per cent glucose to insure initial growth. No disintegration of the cellulose occurred in eighteen days.

Other cultural tests. There is no reduction of nitrates (0.1 per cent) in 0.1 per cent glucose, 1 per cent tryptone broth; nitrites in a concentration of 2 p.p.m. in the same base medium are reduced. Hydrogen sulfide is not produced from tryptone base alone (with 0.1 per cent glucose) but is liberated from sodium thiosulfate (0.25 per cent) and from sodium sulfite (0.1 per cent) in the same base medium, as shown by the lead acetate paper test. The indol test (Gorè method) is negative on both twenty-four- and forty-eight-hour cultures in glucose tryptone broth.

Serological characteristics

Pathogenesis. The cultures are non-pathogenic. Active liver-infusion cultures were fed to white rats with no ill results and intravenous injection of living cultures were given to rabbits with no apparent systemic reaction.

Agglutinin production. As has been previously reported (McClung, 1934) anti-sera of high titer have been prepared for several of the cultures for studies on the agglutination reaction. The specific details of this work will be presented in a later paper of this series.

DISCUSSION

The results of the various tests reported here have usually been discussed as if for one species even though the reactions have been determined with twenty-seven cultures. This has been done since it is believed that the cultures studied represent strains of a single species. Only a few general characteristics are needed for identification and classification. These are preferential growth at thermophilic temperatures, obligate anaerobic

nature, the distinctive microscopic picture of the spore and sporangium, and the extensive saccharolytic (acid and gas produced) and non-proteolytic properties. It is believed that the species has not been previously described and in consideration of the above characteristics, the name *Clostridium thermosaccharolyticum* N. Sp. is proposed.

A review of the literature of thermophilic bacteria has revealed only a few descriptions of obligate anaerobic species, and certain points in the descriptions of some of these may be questioned. Damon and Feirer (1929), reporting the characteristics of four new species, list, in a survey of the literature, only the previous reports of Oprescu (1898), Benignetti (1905), and Bardou (1906) but consider (as does Werkman, 1929) that the organisms isolated by all of these workers have been observed to grow in the presence of free oxygen. Werkman (1929) was not able to confirm the anaerobic nature of one of the cultures, *Clostridium thermoputrificum*, described by Damon and Feirer. Of the cultures of the organisms of Damon and Feirer now existent, which I have been able to obtain, none gives the reactions of the original description. Werkman and Weaver (1927) reported the characteristics of *Clostridium nigrificans*, a thermophilic species, which is non-saccharolytic and only weakly proteolytic. Other reports of thermophilic anaerobic bacilli are those of Veillon (1922), Wollman (1931) and those concerning the anaerobic form fermenting cellulose at thermophilic temperatures, the most recent report of which is that of Snieszko (1933).

If the early reports may be disqualified on the basis that the cultures studied were not obligate anaerobes, and we turn our attention to the later literature we find that separation of the species now described from others is not difficult. The microscopic picture differentiates it from the organisms of Veillon and also from *Clostridium nigrificans*. In each case the cultural reactions also are dissimilar. The same may be said of the cultural reactions of the organism isolated by Wollman and of the four species reported by Damon and Feirer, the terminal spores of which are oval in shape, in contradistinction to the round spore produced by *Clostridium thermosaccharolyticum*. Morphologi-

cally, the species now described resembles the thermophilic cellulose-fermenting type of Snieszko (1933), but separation from this species may be accomplished on the basis of the fermentation of cellulose by Snieszko's organism and its failure to grow in the usual media, in contrast to the reverse reactions obtained with the strains in the present study.

From the more common species of the genus *Clostridium* which possess spherical terminal spores separation may be made on the basis of various cultural reactions, lack of toxin production and preference for growth at thermophilic temperature.

SUMMARY

Morphological and cultural characteristics are given of twenty-seven strains of thermophilic bacilli which are believed to represent the forms responsible for the type of spoilage of non-acid canned foods known as "hard swell." They have been found to be strictly anaerobic and to have an optimum temperature of growth at 55° to 60°C. Morphologically, they are long, slender bacilli possessing a round, terminal spore. The organism is non-proteolytic, extensively saccharolytic, and non-pathogenic. The cultures are believed to represent strains of one species; a review of the literature has failed to reveal a previous description. The name *Clostridium thermosaccharolyticum* N. Sp. is therefore suggested.

These problems concerning the technique of cultivation and the taxonomy of a certain group of thermophilic anaerobic bacteria were begun in the bacteriological laboratories at the University of Texas. To Dr. O. B. Williams and Dr. I. M. Lewis of that University, I wish to express my gratitude for the aid and advice which they gave during the early portion of these investigations. To Dr. E. B. Fred and Dr. Elizabeth McCoy of the Department of Agricultural Bacteriology at the University of Wisconsin I wish to express my appreciation for the suggestions and criticisms which they have given during the latter phases of this work and also for the provision of facilities for its completion. Members of the research staff of the laboratories of the National Canners' Asso-

ciation; namely, Dr. E. J. Cameron and Dr. J. R. Esty, have been most helpful, especially in the supplying of certain cultures and materials and also in the suggestions received during the course of personal correspondence and conference.

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PLATE 1

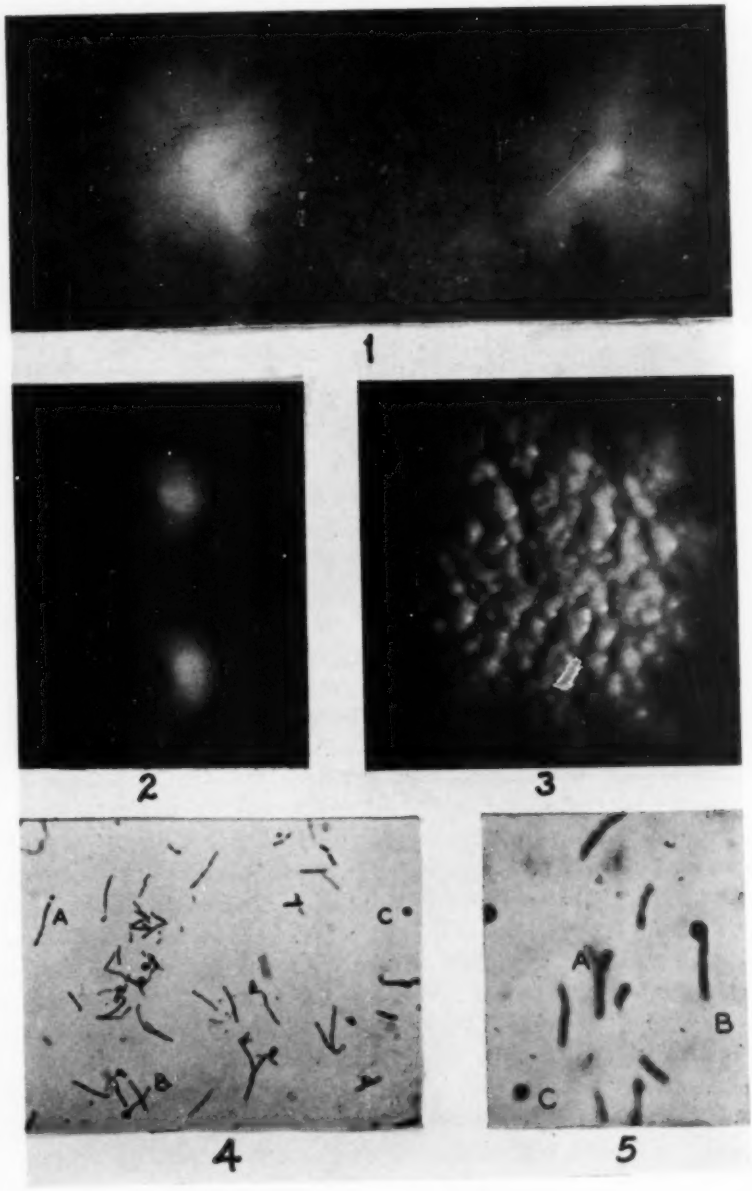
FIG. 1. Typical surface colonies showing slightly raised center and irregular edges.

FIG. 2. Surface colonies of "smooth" (?) type.

FIG. 3. Surface colony showing secondary colonies.

FIGS. 4 AND 5. Photomicrographs showing typical microscopic picture of vegetative cells and spores. In each figure: "A"—vegetative cell; "B"—vegetative cell with immature spore; "C"—mature spore. (The photomicrographs for these figures were taken by Dr. Gorton Ritchie, Department of Pathology, University of Wisconsin Medical School.)

Photographs for figures 1, 2 and 3 were taken of surface colonies on vegetable extract agar. The colonies for figures 1 and 2 had been incubated for ninety-six hours; for figure 3, for twenty-five days. The temperature of incubation was 60°C. and the magnification 8 in each case. The material for the smear for figures 4 and 5 was taken from a surface colony of the same age and type as that shown in figure 1. The slide was stained with dilute gentian violet. The magnification for figure 4 was 1000; for figure 5, 1500 (approximately).



(L. S. McClung: Studies on Anaerobic Bacteria)

STUDIES OF CERTAIN FACTORS INFLUENCING THE SIZE OF BACTERIAL POPULATIONS

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Notwithstanding the wealth of investigation carried out on bacterial growth, relatively few consistent data are available on the intermediate phases; i.e., the phase of negative acceleration and closely related to it, the maximum stationary phase. It would be of interest to know why cells suddenly deviate from an orderly logarithmic growth process to one of progressively increasing generation time and why cells cease to multiply before spatial restrictions limit them.

The literature lacks a convincing trend on any experimental basis. That there are conflicts of opinion and data is not surprising when one considers the vagaries of bacterial strains, the diversity of species requirements, and the difficulty of reproducing in one laboratory the exact experimental conditions in another. The present studies and others to follow are undertaken in an effort to reconcile if possible, by repeating and extending in one laboratory with a single strain of organism, investigations in this field. No special effort is made to review the literature. Henrici (1928) and Rahn (1932) have recently filled that need admirably.

Since the physical factors of environment are subject to fairly accurate control, one may profitably turn to those of biochemical nature.

A strain of *E. coli* has been selected for these studies by reason of the more extensive information available on its metabolism and the ease with which contaminants may be detected. After investigation of various counting techniques the plating method was used. Counting error was consistently within 5 per cent.

Investigation to date has been confined to fluid media. This, unless otherwise stated, was a 1.0 per cent peptone, at a temperature of 37.5°C. Sampling was always preceded by agitation or stirring.

Depletion of food supply, accumulation of metabolites, specific autotoxins, growth-inhibiting hormones and physical crowding have all had their supporters. It is entirely possible, too, that combinations of these may operate.

Relatively little consideration and experimentation seems necessary to render some of these conjectures untenable. In point, is the possibility of physical crowding. Henrici, taking exception to this, has pointed out that organisms growing on solid media are tremendously more crowded than in fluid cultures. Two experiments in these laboratories militate against the crowding theory. A peptone suspension containing 1.5×10^9 organisms killed by exposure to 56°C. in a water bath for one hour, was seeded with a young growing culture of *E. coli*. The control contained a similar number of living coli only. Counts made after fifty hours showed a viable cell count of 100×10^7 in the overloaded culture and 87×10^7 in the control. The slight increase is attributed to nutritives made available by the heat treatment of the killed cells. Certainly, there was no evidence of spatial restriction.

The accumulation of metabolites, the formation of specific autotoxins or the development of growth-inhibiting hormones have received support. Undoubtedly toxic wastes do accumulate. Satisfactory evidence of the latter two has yet to be offered.

In one experiment a culture which had passed its growth peak to a practically stationary phase was divided into two equal portions. To one, was added 0.1 ml. of an extract prepared from peptone by Dr. Sahyun in these laboratories. The other portion served as the control. The initial bacterial population was 44×10^6. At the end of twenty-four hours the control count was 50×10^6 while that in the flask receiving the extract had increased to 100×10^7. The nature or function of this extract has not been fully studied yet. That it is not merely a more readily available form of food serving a nutrition function is definitely established.

It may serve in some way to fulfill an energy need. This weakens the case for specific toxins or growth-inhibiting hormones for these if present, should continue to exert their effect in spite of the activator.

In another experiment *E. coli* was grown in a synthetic medium containing ammonium sulphate and pure amino acids as nitrogen sources. After the culture had passed its peak at 400×10^6 it was divided into two portions. To one was added 1 cc. of 2 per cent peptone and to the other an equivalent amount of nitrogen as synthetic medium. Counts after twenty-four hours showed a population of 800×10^6 per cubic centimeter, in that receiving peptone and 490×10^6 in that receiving the synthetic medium.

It cannot be denied that the accumulation of metabolites acts to restrict bacterial activity. But the rôle of the acknowledged toxic wastes in many cases has not been satisfactorily disengaged from other "possible factors." That is, certain phenomena may operate to restrict growth before wastes become significant. The effect of toxic substances present might then be enhanced. Depletion of food, exhaustion of energy sources or both might conceivably be such factors.

Some evidence in this direction is offered by an experiment in which 1.0 ml. of 10 per cent peptone was added daily to a culture whose initial volume was 100 ml. Additions were begun fifty-four hours after the inoculation when the culture had passed its growth peak of 124×10^7 and declined to 107×10^7. The results with the control are included in table 1. In a similar experiment, half the volume was withdrawn each day and replaced with 1 per cent peptone. These data confirm those of Graham Smith (1920). It would appear that depletion of food, or the ratio of some essential constituent to the number of cells, might be concerned with retardation or cessation of growth. In any event, the evidence points to the fact that growth is retarded by some factor other than growth toxins. The diminution of toxicity was not due simply to a buffering action of the peptone. The renewal of food supply or some vital constituent remains open.

When the culture was aerated at intervals, the rate of growth was appreciably accelerated and the twenty-four-hour crop

approximately doubled. Following, however, ensued a rapid drop in the aerated culture while the control culture continued a slow increase so that the populations in the two approximated each other in thirty-six to forty-eight hours. Further aeration resulted in unimportant changes. This again might indicate depletion of some vital constituent, but the present knowledge of bacterial requirements and peptone composition stand in the way of proving or disproving this possibility. There is the further

TABLE 1

Effect of addition of concentrated nourishment to old cultures

GROWTH PERIOD	ADDITION OF MEDIUM	CONTROL	PARTIAL REPLACEMENT	CONTROL
hours				
0	2.3*	2.3*		
24	1,240	1,240	1,900*†	1,900*
75	700	760	1,600†	900
80	†			
96	1,280†	720	1,800†	700
125	1,220	630	1,500	400
131	†	410		
145	500			
155	†			
170	400†	112		
193	510†			
216	400†	9		
230	180			

* Cells per milliliter (in millions).

† Peptone added.

possibility of some volatile inhibiting substance being removed by the aeration. This likewise is difficult to establish satisfactorily, yet when nitrogen was used instead of air with restoration of the air environment after bubbling no significant increases in populations were noted. There remains for consideration the effect of the aeration on the oxidation-reduction potential of the system and this will be considered in a later paper. A system whereby food could be renewed at any desired rate and the accumulations of wastes prevented might provide a means of evaluating more satisfactorily the significance of the various controlling factors.

The device adopted by Rogers (1930) suggested a means of approaching such conditions. The arrangement shown in the diagram was developed and utilized in further studies. Broth flows from a reservoir through a capillary into a 500 ml., three-necked flash which serves as the culture chamber. An automatic siphon maintains a constant volume in the chamber, discharging

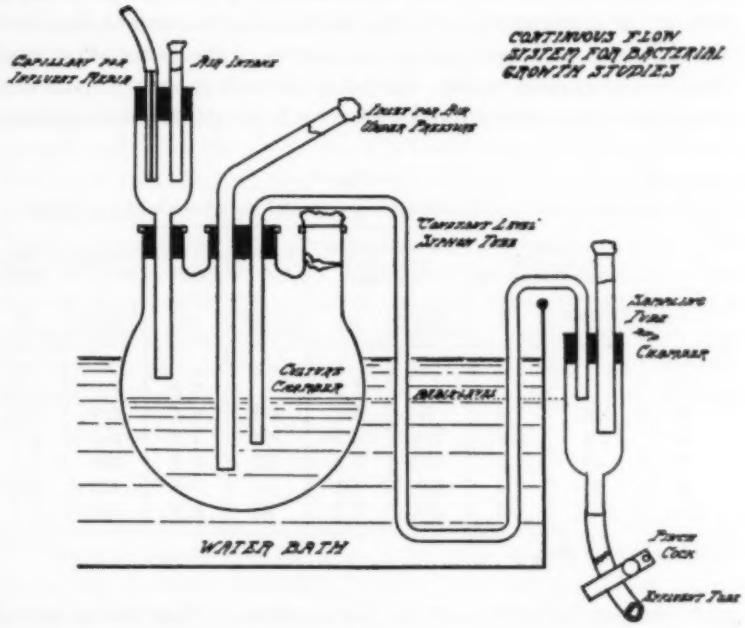

FIG. 1

the overflow into sterile bottles. Rate of flow can be varied from 1.0 to 500 cc. per twenty-four hours by changing the level of the food reservoir. Rate of change in the culture chamber may be controlled by varying the volume of the culture. The organisms are virtually growing in a constant stream of fresh food. Wastes are at least maintained at a significantly lower level than in the ordinary stationary culture. The effect of varying peptone concentrations, of glucose as an additional energy source both in

buffered and unbuffered broths, and of aeration by means of a motor driven stirrer were investigated in this continuous-flow set-up.

The effect of the continuous flow arrangement was very apparent. In a stationary culture the count rose to 189 $\times$ 10^7 in twenty-four hours and decreased to 140 $\times$ 10^6 after one hundred sixty-eight hours. In the continuous-flow the count rose to 140 $\times$ 10^7 in twenty-four hours and was maintained at that level through one hundred ninety-two hours. The population peak attained is approximately the same in both cases. In the constant flow more time is required to reach this peak since organisms

TABLE 2

Effect of glucose and continuous flow on size and maintenance of population

TIME	STATIONARY CULTURE—NO GLUCOSE UNBUFFERED		CONTINUOUS FLOW NO GLUCOSE UNBUFFERED		STATIONARY 0.25% GLUCOSE BUFFER		CONTINUOUS 0.25% GLUCOSE BUFFERED	
	Cells per milliliter*	pH	Cells per milliliter	pH	Cells per milliliter*	pH	Cells per milliliter*	pH
hours								
0		7.2		7.2		7.2		7.2
24	128	7.5	79	6.7	174	6.4	228	6.5
48	107	8.0	115	6.9	227	7.3	315	6.9
72	63	8.2	130	7.1	137	7.5	370	7.1
96	40	8.4	190	7.1	83	7.6	325	7.0
120	17	8.7	190	7.1	63	8.2	320	7.1

* Times 10 million.

are continually being lost to the overflow. That mere lack of food was not the only factor is evidenced by the fact that when the culture was filtered and reseeded a population of 600 million was reached. This observation is of course not new. At the end of one hundred ninety-two hours, glucose was added to the constant flow to give an approximate concentration of 0.25 per cent and in twenty hours the population had jumped to 195 $\times$ 10^7. A severe drop followed, due probably to the drop in pH to 5.3.

The next experiment indicated the effect of glucose and pH changes more clearly. Observations on 1.0 per cent peptone cultures containing 0.25 per cent glucose buffered with M/50 phos-

phate in both stationary and continuous flow set-ups, in contrast with a stationary peptone culture without glucose, are shown in table 2.

In this experiment as in the others the effect of waste accumulations is apparent. The pH effect is well correlated with maintenance of the population. This is, of course, especially noticeable when the sugar media are unbuffered.

In this experiment, too, there are suggestions that some other factor than toxicity is acting to restrain growth. The effect of food additions makes this evident. That it is not necessarily mere lack of nourishment in form of cell-building constituents may be inferred from the experiment before cited in which old broth was re-inoculated, and also from the effect produced by adding a readily available form of energy such as glucose. When this is added there is not only an increased growth rate, but the final populations are significantly higher, and under conditions where the accumulations of wastes are prevented and both food and energy are continuously renewed this high population level may be maintained indefinitely. The effect of glucose is significantly indicated by comparing two constant flow chambers, one of which carried glucose and the other merely peptone.

DISCUSSION

The inference may be drawn that growth is restrained first by a change in the ratio of cells to all building material. That food requiring the least amount of energy for reorganization is first utilized. With these compounds exhausted or reduced, constituents requiring more energy for reorganization are utilized, but since more energy is required and less per cell available, the bacterium becomes less efficient and growth is checked. Possibly at this point the effect of the metabolites on the cellular enzymatic activities becomes apparent and the weakened cells die.

The above speculations gain credibility by recalling the studies of Clifton (1933) on "resting bacteria." It was pointed out that while the rate of reduction of ferricyanide increases with concentration of peptone up to concentrations of 2.0 to 3.0 per cent, further increases in concentration bring reductions in the rate.

Likewise it has been noted in the present studies, that higher concentrations than 2.0 per cent peptone fail to increase significantly the numbers of cells. Changes in hydration and viscosity of the substrate probably begin to play an important rôle beyond this point, although data are not available. Clifton further points out that the rate of reduction per cell per minute of ferricyanide in a 1.0 per cent peptone solution is reduced with increasing numbers of cells, and increases as the concentration of the oxidant is increased. Those data may be regarded as the resultant of bacterial activity—the intensity and duration of which will determine the population at any interval.

In the normal growing culture of organisms concentration of cells is continually increasing and concentration of oxidant decreasing. Consequently a decrease in bacterial activity as reflected by numbers of cells is to be expected. Even at this point growth may be again accelerated by the addition of available energy. This cannot be long supplied by peptone alone. More readily available energy in the form of glucose brings about further increase in numbers, continuing until the maximum efficiency for the amount of energy, the type and amount of cell building material is reached. This will logically vary with the species. Increase of cells cannot further occur unless energy is introduced which is more easily available or some other method of increasing enzymatic efficiency is invoked.

SUMMARY AND CONCLUSIONS

An apparatus for studying growth phenomena in a constant flow is described.

Physical crowding is unimportant in restraining bacterial growth.

The formation of specific growth-inhibiting substances is discounted by the experiments dealing with growth acceleration of cultures in the phase of decline.

The effect of metabolites is a variable, depending on the metabolite and its effect on the enzymatic mechanism of the cell.

Growth will not mount above a certain point even with the

effect of metabolites reduced to a low level. We believe this to be because of the reduction in energy available per cell.

Growth ceases because of changes in the availability of nutrient material and energy demand.

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SOME PROPERTIES OF A BACTERIAL-INHIBITORY SUBSTANCE PRODUCED BY A MOLD[1]

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In 1929, Fleming observed that a mold which he identified as *Penicillium rubrum* (Biourge), but which more closely resembles *Penicillium notatum* (Westling) and belongs to the group of which *Penicillium chrysogenum* is the type species, (Thom, 1930) was able to produce a substance which inhibited the growth of certain bacteria. During 1930 a large number of molds[2] were studied in this laboratory in an attempt to find others than the one isolated by Fleming which would produce a similar inhibitory substance. Since none were found, the following experiments were conducted on the filtrate from Fleming's culture when grown on veal infusion broth or on a modified Czapek-Dox synthetic medium having the composition described by Clutterbuck, Lovell and Raistrick (1932) with the hope of gaining some information with regard to the nature of the inhibitory substance.

The mold was permitted to grow at 20°C. for a period varying from seven to ten days on veal medium or twenty to twenty-five days on the synthetic medium. After the growth period, the

[1] Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station, June 11, 1934, as Technical Paper No. 657.

[2] The molds studied were collected from various sources and identified by Dr. Chas. Thom, U. S. Department of Agriculture. They include the following: *Mucor mucedo, Mucor piriformis, Rhizopus nigricans, Aspergillus glaucus, A. nidulons, A. niger, A. flavus, Penicillium expansum, P. roqueforti, P. digitatum, P. illaticum, P. rubrum* (Biorge) according to Fleming, *P. cephalotherium roseum, P. cladosporum herbarum, P. hormondendrum cladosporoides, P. notatum* (Westling), *P. rubens, P. chrysogenum, Oidium lactis, Fusarium oxysporum* and three species of Actinomycetes.

mold was separated from the substrate by filtration through a Berkefeld filter, leaving a sterile filtrate. The filtrate containing the inhibitory principle had a bright yellow color. This pigmentation regularly occurred soon after fructification and became deeper, approaching a red orange, if allowed to incubate for a long period of time. The filtrate was tested for inhibitory substance in the following manner: Into one-half of a Petri dish was poured nutrient agar without the filtrate; into the other, the same agar plus filtrate. The plates were then streaked with the cultures of bacteria. Growth on the half of the plate containing the nutrient agar only was evidence of no error in streaking. Absence of growth on the other half indicated the presence of the inhibitory substance.

Among the organisms that are inhibited by the filtrate are *Staphyloccocus aureus* (which became the test organism), *Staphylococcus citreus*, *Staphylococcus epidermidis*, a pneumococcus, a hemolytic streptococcus, *Bacillus anthracis*, *Corynebacterium diphtheriae* and *Streptococcus pyogenes*. The organisms not inhibited include *Bacillus subtilis*, *Escherichia coli*, the typhoid and paratyphoid organisms, *Pseudomonas fluorescens*, *Bacillus influenzae* and *Bacillus proteus*. An investigation of the Gram's reaction, physiological and cultural characteristics of the inhibited organisms reveals no characteristics consistently common to this group, nor was there any characteristic which differed uniformly from those of the organisms not inhibited. The potency of the inhibitory substance is indicated by the fact that a 1:100 dilution of filtrate in nutrient medium was found sufficient to inhibit the sensitive organisms. In some instances dilutions of 1:800 brought about complete inhibition.

Effect of inhibitory substance on bacteria. In order to determine if inhibition is due to a lysing of the organism, as stated by Fleming (1929), tubes containing 10 ml. of nutrient broth and varying amounts of the filtrate were inoculated with *Staphylococcus aureus* and incubated at 37°C. Streaks were made from tubes showing no apparent growth at the end of twenty-four hours. Normal growth was obtained on all plates. From this it is concluded that the organism is not destroyed but merely inhibited.

Effect of light. That light has a deleterious effect upon the production of the inhibitory material was shown by subjecting the actively growing culture to the rays from a carbon arc lamp with a quartz filter, giving rays including the longer ultra violet, infra red and other light rays. The cultures were grown in Pyrex glass flasks[3] and exposed to these rays for a period of fifteen minutes daily for the period of incubation. Caution was exercised to prevent contamination during exposure and to prevent the action of heat from the lamp upon the culture. Filtrates from cultures so treated contained no inhibitory substance, showing that the inhibitory substance was either not produced or if produced, was destroyed by the action of the light rays.

Effects of oxygen, hydrogen and carbon dioxide. Since oxygen plays an important rôle in the production of toxins, it was decided to increase the concentration of that gas in the substrate upon, and in the atmosphere in which, the molds were growing. This was accomplished by bubbling oxygen through the medium at the rate of about 100 bubbles per minute for fifteen minutes each day. The flasks were stoppered with 2-holed rubber stoppers through which passed glass tubes equipped with rubber tubes and pinchcocks. In this way oxygen was passed through the substrate and an atmosphere of oxygen maintained above the medium at all times. The gases used in this work were obtained in tanks from the Ohio Chemical Company. The presence of oxygen produced no noticeable effect on the amount of growth or pigment formation although no inhibitory substance was produced under these conditions. This was contrary to expectations and would indicate a dissimilarity between the inhibitory substance and a true soluble toxin because it was either not produced, or if produced was destroyed by the action of the gas.

Cultures were treated in the same way with hydrogen and carbon dioxide. The results were identical with those obtained when an oxygen atmosphere was maintained.

Effect of temperature on stability of inhibitory substance. The

[3] According to the Corning Glass Works, Manufacturers of Pyrex, the type of glass of which these flasks are made permits "medium ultraviolet transmission, and is highly transparent to visible radiation. . . . . "

filtrate was found to be active after heating at 80°C. for seventy-five minutes and at 90°C. for fifty-five minutes. Heating at 90°C. for sixty minutes destroyed its activity. Heating at 100°C. for five minutes did not destroy the inhibitory activity but after ten minutes at this temperature the filtrate was no longer active.

Attempts to isolate the inhibitory substance by distillation. Attempts to separate the inhibitory substance from the mold filtrate by means of distillation at low temperatures under reduced pressure resulted in a complete loss of the inhibitory material as it could not be recovered from either the distillate or the residue. In this distillation a vacuum pump was used to obtain a reduced pressure sufficient to cause boiling at 42°C. to 52°C.

Attempt to separate the inhibitory substance by dialysis. In repeated experiments, using samples of the active inhibitory filtrate placed in collodion bags and allowed to dialyze into sterile distilled water for thirty-six hours, a considerable amount of the yellow pigment passed into the dialysate, apparently setting up an equilibrium between the dialysate and the residue. Both the dialysate and residue were tested for inhibitory properties with negative results. The combined dialysate and residue showed no inhibitory power.

Relation of pigment to inhibitory substance. Since the pigment and the inhibitory substance usually appear simultaneously, an attempt was made to determine whether or not the pigment is the inhibitory substance or related to it. By adsorption on charcoal the pigment and the inhibitory substance are both removed. This was the only evidence obtained that would indicate a close relationship between the two. On the other hand, chloroform extraction removed some of the pigment without affecting the intensity of the inhibitory properties of the broth filtrate. Control experiments showed that the inhibition was not due to traces of chloroform left in the broth. Continuous extraction with ether and acetone for seventy-two hours failed to remove either the pigment or the inhibitory substance. Holding of the broth filtrate containing the inhibitory principle at room temperature, in the dark, at a pH of approximately 8.0 for three to four weeks resulted in a loss of the inhibitory activity without any

decrease in pigmentation. It has been shown that exposure to light does not allow the production of the inhibitory substance while color production is unaffected. Likewise, oxygen, hydrogen and carbon dioxide prevent the production of the inhibitory substance and do not affect pigmentation. Heating brings about a loss of inhibitory activity with no loss in the intensity of the pigment. Thus it seems that there is no close relationship between these two.

Enzymes present in an active filtrate. That enzymes are associated with normal mold growth has been shown by numerous investigators. In order to obtain more information with regard to the nature of the active filtrate, it was thought desirable to determine what enzymes were present. Tests indicated the presence of erypsin and catylase in large amounts. Lipase was present in moderate amounts while traces of amylase, trypsin and amidase were found. Tests for gelatinase and emulsin were negative.

TABLE 1

Surface tension readings on non-inhibitive and inhibitive media

SYNTHETIC MEDIA BEFORE MOLD GROWTH	INHIBITORY FILTRATE FROM SYNTHETIC MEDIA AFTER MOLD GROWTH	NUTRIENT BROTH + 10 PER CENT FILTRATE-INHIBITED STAPHYLOCOCCUS AUREUS
52.6 dynes	43.1 dynes	46.25 dynes

Effect of filtrate upon charge of bacteria inhibited. Experiments to determine the degree of charge on *Staphylococcus aureus* as influenced by the inhibitory substance were performed. Suspensions of the organisms were made in nutrient broth, in the inhibitory filtrate active in 1:500 dilution, in veal infusion broth and in synthetic broth. The charge on the organism was measured electrophoretically. The degree of charge was found to be the same in each case indicating that the inhibitory substance does not alter the charge on the organism which it inhibits.

Surface tension. As indicated in the literature (Albus and Holms, 1926; Davis, 1927; Gibbs, Batchelor and Lickels, 1926; Kopeloff and Beerman, 1926, 1927; Pizarro, 1927) a lowered sur-

face tension may have an inhibiting effect upon bacteria, especially upon *Staphylococcus aureus* and *Corynebacterium diphtheriae*, both of which are readily inhibited by the filtrate from Fleming's mold culture. Samples of nutrient broth, nutrient broth plus filtrate, undiluted filtrate and synthetic medium were tested. The results obtained after many readings on the Cenco-DuNoüy tensiometer are given in table 1. It seems unlikely that this reduction of surface tension is sufficient to cause the marked inhibitory effect noted.

SUMMARY

1. The production of an inhibitory material by molds is not common. Only one of many species studied produced it.

2. The inhibitory substance studied is selective in its action, affecting relatively few bacteria and these of types apparently not closely related.

3. The pigment produced by Fleming's mold is not identical with the inhibitory material.

4. Light, oxygen, hydrogen and carbon dioxide prevented the production of the inhibitory material or destroyed it.

5. The inhibitory substance is relatively thermo-stable.

6. The inhibitory substance is not obtained free from the rest of the filtrate by dialysis, adsorption or distillation at low temperature.

7. The charge upon the bacteria inhibited by the mold filtrate is not changed by the filtrate.

8. Several enzymes were found present in the filtrate containing the inhibitory substance.

9. The surface tension of the broth upon which Fleming's mold culture is grown is decreased slightly.

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THE PIGMENT OF BACILLUS VIOLACEUS

I. THE PRODUCTION, EXTRACTION, AND PURIFICATION OF VIOLACEIN[1,2]

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In the course of an investigation, in progress since 1929, on the chemistry of violacein the violet pigment of *Chromobacterium violaceum* (*Bacillus violaceus*), it became necessary to devise a method for the preparation and purification of the pigment in considerable quantities.

Lasseur and Girardet (1926) prepared and purified a small quantity of violacein by culturing a violet bacillus on Lasseur's synthetic L_1 medium containing asparagin; with a subsequent extraction, washing, and purification of the pigment, which was then subjected to a spectroscopic examination. Reilly and Pyne (1927) isolated 60 mgm. of the pigment by growing *B. violaceus* for three weeks on 6 liters of lactose broth at 20°C., filtering out the pigment, drying, extracting with alcohol, concentrating, precipitating with water, and washing with chloroform. An ultimate analysis and a molecular weight determination were made on the product. Friedheim (1932) studied the effects of the pigment upon the respiration of living cells. He prepared it from cultures of *B. violaceus* grown upon solid media, by extraction with acetone, which was then evaporated, the residue washed with water, dissolved in dry ether, and precipitated with HCl gas as violacein hydrochloride. The pigment was obtained in

[1] Extract from a thesis submitted to the Graduate School of the Massachusetts Institute of Technology, Cambridge, Massachusetts, October 17, 1933, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

[2] Contribution No. 39 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Massachusetts.

the free state by hydrolysis of the hydrochloride with water. While the present paper was being prepared for publication, an article on the chemistry of violacein appeared by Wrede and Rothhaas (1934). They give no method for culturing the organism but promise that they will publish their method shortly.

EXPERIMENTAL

After numerous trials with various solid and liquid culture media, the following method of culturing was adopted. Standard lactose broth, containing 5 grams lactose, 5 grams Armour's peptone, and 3 grams Liebig's beef extract per liter of water, was prepared in a 10-liter lot and distributed between four 3- or 4-liter Florence flasks, each of which was equipped with a two-hole rubber stopper bearing a glass oxygen-supply tube reaching nearly to the bottom of the flask and drawn out to a fairly fine tip, and a short oxygen-exhaust tube reaching only a short distance below the stopper. Each supply tube was equipped with about a foot of tight-fitting rubber pressure tubing. The stoppers were inserted loosely in the flask mouths and the flasks were sterilized at 20 pounds pressure for twenty minutes. The stoppers were then thrust firmly into the flask mouths and the broth allowed to cool overnight. The pH of the broth was now approximately 6.20 by the quinhydrone electrode.

One to 2 cc. of a twenty-four- to forty-eight-hour broth culture of *B. violaceus* was now inoculated into each flask, the flasks were connected in series, and the stoppers were fastened firmly in position by means of copper wire. The first flask of the series was then connected to an oxygen supply and a current of oxygen allowed to bubble through the broth at a rate of about 170 cc. per minute. A lesser flow of oxygen gave a slower growth and pigmentation by the bacillus; a greater flow produced much spattering of the broth. Growth and pigmentation reached an apparent maximum after six days at room temperature (22 to 27°C.), the broth in all flasks being turbid and intensely violet due to the insoluble pigment produced. On microscopic examination some of the pigment appeared to be intracellular, but most of it was in irregular masses outside the cells.

The pigment was precipitated from suspension by placing the broth, which now had a pH of about 7.00, in one or more tall cylinders, adding 14 to 20 cc. of glacial acetic acid and 4 grams of kallite (diatomaceous earth) per liter, stirring well, and allowing the mixture to stand overnight. To determine the exact amount of acid needed for each lot of broth, tests were run with varying amounts of acid upon small measured samples of broth, and the proportions giving optimum precipitation were then used on the main portion. The most complete precipitation usually took place at pH 3.65. When sedimentation of the pigment was complete, a violet deposit appeared at the bottom of the cylinder, while the supernatant was turbid and brown in color, with usually no more than a tinge of violet. Many of the bacteria remained in suspension. The supernatant, amounting to about 95 per cent of the total volume, was removed by suction or decantation.

The dark violet sludge of pigment, kallite, and bacterial cells, was poured upon 24 cm. folded filters, allowed to drain, and then washed with water. While still moist, the filter bearing the precipitate was placed in a 43 by 123 mm. extraction thimble in an electrically heated all-glass Soxhlet extractor and extracted with 95 per cent ethyl alcohol. Extraction was complete after about fourteen hours. The extract was concentrated to about 50 cc. in the extraction flask and the pigment precipitated by adding about 250 cc. of distilled water in a fine stream. Slow precipitation could be speeded by adding 3 to 4 per cent by volume of glacial acetic acid. The pigment was then filtered out, washed with water, and dried. On microscopical examination, small light colored particles could be seen among the dark masses of pigment and a greasy streak was produced when the preparation was rubbed across glass. To remove these lipoidal impurities the pigment was successively soxhletted for six to twelve hours each with redistilled petroleum ether, chloroform (free of water and alcohol), and redistilled carbon tetrachloride. The petroleum ether and carbon tetrachloride extracted a considerable amount of brown material, while the chloroform took on a faint pink color due to a trace of the pigment going into solution or suspension. The pigment was then reëxtracted with alcohol, repre-

cipitated with water, filtered on a hardened filter with suction, and dried. The last extraction with alcohol was prolonged by the fact that the pigment redissolved only very slowly. About 250 mgm. of a highly concentrated, lipoid-free preparation of violacein was obtained by this process from each 10 liters of broth. It is a dark violet, almost black, amorphous fine powder, which does not melt at 300°C., but decomposes without fusing at a higher temperature.

DISCUSSION

Oxygenation of the cultures greatly reduced the time required for maximum pigment production. Pigmentation appeared to be heavier if cellulose, in the form of weighing paper or filter paper, was not allowed to come in contact with the broth. The nature of the peptone used was of great importance. Armour's peptone or Digestive Ferments Company, proteose-peptone gave excellent pigment production, while Difco or Witte peptones gave abundant growth but little pigmentation. The addition of kallite aided both the precipitation and the subsequent alcohol extraction of the pigment. Extracting the mass while still moist also speeded extraction, since the alcohol seemed to penetrate the wet material more readily. All-glass apparatus was used to avoid contamination from tinfoiled cork or rubber stoppers.

Despite repeated trials, the pigment was not obtained in a crystalline condition. Wrede and Rothhaas (1934) subsequently obtained needle-like crystals from pyridine solutions of the pigment and claimed that these represent pure violacein.

SUMMARY

1. By the passage of oxygen through cultures of *B. violaceus* in lactose broth, a heavy growth and pigmentation is produced after six days at 22 to 27°C.

2. Armour's peptone or Difco proteose-peptone give excellent pigmentation, while Witte or Difco peptones give very little.

3. The pigment, violacein, is precipitated in a crude state by adding kallite and glacial acetic acid to obtain a pH of about 3.65, and allowing sedimentation to take place over night.

4. The pigment is extracted with alcohol, precipitated with water, washed successively with petroleum ether, chloroform, and carbon tetrachloride; it is redissolved in alcohol, reprecipitated with water, and dried.

5. Two hundred and fifty milligrams of violacein, free of lipoids, is obtained from 10 liters of broth.

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A NEW SPECIES OF THE GENUS BACILLUS EXHIBITING MOBILE COLONIES ON THE SURFACE OF NUTRIENT AGAR

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During an investigation of the intestinal flora of several termites from central Texas, a characteristic bacterial species was frequently observed. The uniformity of its occurrence seems to indicate that this organism is a normal inhabitant of the termite intestine. Isolation was accomplished by dissecting the intestine, mashing its contents into a drop of sterile water, and streaking the diluted excreta over the surface of nutrient agar. Subsequent studies on pure cultures have shown that the organism is not identical with any which has been found previously reported in the literature. It appears to be an undescribed species belonging to the genus *Bacillus*. The following are the principal diagnostic characteristics of the organism:

CULTURAL CHARACTERISTICS

Nutrient agar plate. The growth of the organism on the surface of nutrient agar is only moderate, even under the best conditions for cultivation. Young colonies are translucent and watery. Due to certain characteristic behavior of young colonies, a more detailed description of their sculpture is rendered impossible. Older colonies (three to five days) are slightly opaque and of butyrous consistency.

The outstanding characteristic of the organism is the mobility of young colonies on the surface of nutrient agar entirely free from excess moisture. This mobility expresses itself in two characteristic forms. In certain colonies a clearly visible rotary motion occurs, whereas in other cases the entire colony, consisting

of thousands of cells, may exhibit a migratory mobility, moving over the agar at the rate of 0.01 mm. per second. Although the movement of such colonies is not visible to the unaided eye, in later stages the colonies and the paths over which they have traversed may be seen without the aid of a lens (fig. 1).

In order to determine the nature and cause of mobility, the series of morphological changes undergone by the colony during its development was studied in detail. An agar plate was inoculated from a glucose broth culture of the organism in the maximum growth phase. The colonial development was observed with the low power objective (× 150) at ten-minute intervals for the following thirty hours.

After five hours of incubation the colonies were microscopic, each consisting of a single-layered, somewhat loosely arranged plate of cells. After about seven hours the first indications of motility appeared. The initiation of movement in different colonies was almost simultaneous, practically all the colonies being somewhat mobile after seven and one-half hours. The cells of a non-mobile colony could not be made to move by smearing the edge of the colony with a probing needle. Movement within the colony usually began at a short distance inward from the periphery. With few exceptions the cells at the outer boundaries of the colony were non-motile. In some cases this periphery was never broken, movement being limited to the interior. In this event motility expressed itself first through unorganized slowly milling masses of cells which finally synchronized their motion and produced either levo- or dextro-rotary plates, the cells of which changed their relative positions but little or not at all (fig. 4). The rotation caused the colonies to become somewhat rounded.

In the majority of cases the periphery was broken by the moving cells before a round colony was formed. The release of the cells from the inner portion allowed the colony to move over the agar with a rapid migratory mobility. Such colonies assumed the shape of a bullet with a round but somewhat pointed anterior portion and a deeply concave posterior portion. From the edges of these colonies cells were constantly being lost, thus forming a

"colony track" composed of two parallel streaks seeded by the moving colony (fig. 2). The cells which were detached from the mother colony immediately became non-motile; a mass of cells seemed necessary for motility. Subsequent growth of the detached organisms made the tracks clearly visible during later stages of growth (fig. 1). Migratory colonies always described arcs, or spiral paths until the cell mass finally stopped and rotated in the center of the described spirals (fig. 2). Smaller sub-colonies sometimes broke from the mother colony and moved over independent courses.

After eighteen hours the rotating colonies were composed of numerous concentric plates of cells stacked upon each other in pyramidal fashion (fig. 3). After thirty hours the motility had ceased. After forty hours the surface of the colonies became smooth or slightly contoured, rising above the agar in helicoid fashion.

Sub-surface colonies show no tendency to be mobile and are ellipsoid, often with a fuzzy growth at one end. Various consistencies of agar have been tested and the organism has been found motile in every case. The agar is never liquefied.

No entirely satisfactory explanation for the mobility of the colony is offered. Observation of the morphological changes undergone by the colony has yielded insufficient evidence to warrant definite conclusions. The nature of the movement would indicate that it is due to the action of the flagella within a watery secretion produced by the cells themselves. The unique character, or combination of characters, which enables this organism to exhibit mobility on dry solid media, exclusive of other motile, mucous-forming species, has not been determined. It is possible that the cells become entangled by their flagella to such an extent as to form a more or less connected cellular mass similar to such colonial forms as Volvox or Pandorina. Mere entangling of the flagella would serve to explain the tenacity with which the colony is held together during migration but would not in itself explain the unified synchronism of motility. We offer no explanation for the harmony which exists between the moving cells but merely call attention to the fact that when the organisms are held in

close proximity as they are on the surface of nutrient agar, maximum inter-cellular influences are exerted.

The rotary movement and the spiral migratory mobility of the colony as a whole are in harmony with an unexplained tendency toward spiral growth and motion which occurs throughout nature. The common occurrence of spirality in animals and plants tends to support the view that it is due to something fundamental in organized matter.

Plain broth. Growth in nutrient broth after twenty-four hours of incubation is moderate with an even clouding. Older cultures have a delicate pellicle and a slightly viscid sediment.

Agar slope. The growth is moderate, spreading and translucent.

Gelatin. Gelatin is completely liquefied after thirty days incubation.

Litmus milk. There is a slight reduction of the litmus in the bottom of the culture tube after ten days of incubation. Otherwise no change is noticeable.

PHYSIOLOGICAL CHARACTERISTICS

Fermentation. Peptone water was adjusted to pH 7.2, sterilized, and various carbohydrates in distilled water solutions were added in 0.5 per cent amounts under aseptic conditions. A stock strain of the organism, which had been in culture for three months was used in the tests. An alkaline reaction was produced in all the carbohydrate media tested. These reactions have been consistent on repeated tests. Quantitative tests for the presence of glucose indicate that this carbohydrate is utilized without acid production. Starch-agar plate cultures of the organism, flooded with iodine after four days of incubation, gave no indication that starch is hydrolyzed. Cellulose is not decomposed. No acid is produced from mannitol, sorbitol, adonitol or dulcitol.

Indol production. No indol was present in seventy-two-hour tryptophane broth cultures tested by the method of Goré.

Production of hydrogen sulfide. Hydrogen sulfide is not produced in lead acetate agar stabs as indicated by the failure of the medium to blacken within ten days.

Reduction of nitrates. Cultures prepared on nitrate agar slopes and subsequently tested for the presence of nitrites by the sulphanilic acid-naphthylamine method showed that nitrates are not reduced to nitrites.

Temperature relations. The organism grows best at 22°C.; growth and sporulation are markedly inhibited at 37°C. There is no motility at 37°C.

Oxygen relations. Best growth occurs under aerobic conditions. Some growth was obtained, however, under anaerobic conditions produced by oxidising phosphorus in a sealed jar.

MORPHOLOGY

Vegetative cells cultured on nutrient agar at 22°C. for twenty-four hours are from 3 to 5μ in length by 0.5μ in diameter. The cells occur singly and in short chains. The ends of the cells are rounded. Terminal endospores are formed after forty-eight hours growth on nutrient agar; swellings on the vegetative cells at the site of spore formation indicate the initial steps in sporulation (fig. 5). The mature spore is quite large, almost entirely filling the swollen sporangium, and is spherical to ovoid in shape.

In liquid media very active motility is accomplished by means of numerous peritrichic flagella.

The Gram reaction is negative in cultures varying in age from 9 to 36 hours when stained by that variation of the Gram stain suggested by Kopeloff and Cohen (1928). The bacteriostatic action on the organism, however, is typical of that on Gram-positive forms, growth being inhibited in crystal violet diluted 1:200,000. We realize that Gram-negative, spore-forming aerobes are unusual if they occur at all. Since Gram-variable forms have been shown to exist, and in view of the atypical bacteriostatic action, we hesitate definitely to characterize this organism as Gram-negative in spite of its consistency with regard to the technique employed.

DISCUSSION

This organism does not seem to agree in all particulars with any species heretofore described. According to Bergey's Manual

(1934) there are only eight aerobic, facultative, mesophilic, motile bacilli with terminal spores which cause a swelling of the vegetative cell at sporulation, and which do not produce a yellow pigment. The organism is definitely different from these eight. It differs from all the described species within this group with regard to optimum temperature and the mobility of colonies on the surface of nutrient agar. The characteristics of the organism are not in agreement with those of any of the eight, even with regard to the more salient characteristics. In view of these facts introduction of this organism as a new species is believed justified and the descriptive name *Bacillus rotans* is suggested. Cultures of this organism have been placed in the American Type Culture Collection.

While many of its characteristics, particularly the mobility of colonies, are strongly suggestive of the *Myxobacteriales*, we believe that the organism is a true bacterium. Failure to produce fructifications after long incubation and the ability to form flagella and endospores are evidence for placing the organism in the genus *Bacillus*.

RÉSUMÉ

Bacillus rotans, n.sp.

A medium sized rod with rounded ends, occurring singly and in short chains; actively motile by means of numerous peritrichic flagella; characteristic mobility of colonies on the surface of nutrient agar; terminal spores which cause a marked swelling of the vegetative cell at sporulation; no pigment produced; gelatin liquefied after thirty days; no coagulation or digestion of milk, but reduction of litmus; no hydrogen sulfide or indol formed; nitrates not reduced; alkaline in all carbohydrate media; optimum temperature 22°C.; oxygen relation, aerobic preference; Gram-negative but sensitive to dilute crystal violet.

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KOPELOFF, N., AND COHEN, P. 1928 Stain Tech., 3, 64.

PLATE

PLATE 1

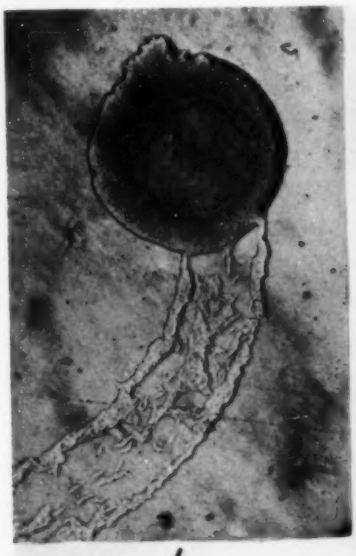
FIG. 1. Old migratory colony after the period of active mobility, showing growth of detached cells in the path of the colony. × 150.

FIG. 2. Migratory colony in active mobility, showing the spiral path of the colony. × 150.

FIG. 3. Old rotating colonies trapped in a mass of cells, showing concentric plates of cells stacked in pyramidal fashion. × 150.

FIG. 4. Typical rotating colony after twelve hours growth on nutrient agar. × 150.

FIG. 5. The individual cells after forty-eight hours growth on nutrient agar, showing the swollen sporangium. × 1500.



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(J. Lemoyne Roberts: New Species of Genus Bacillus)

THE SIGNIFICANCE OF MARINE BACTERIA IN THE FOULING OF SUBMERGED SURFACES

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The attachment and subsequent growth of the wildly promiscuous assemblage of visible plant and animal organisms on the hulls of ships and other submerged marine structures is known as fouling. Our present-day knowledge of the nature and extent of fouling is discussed comprehensively by Visscher (1928b). The vast economic loss resulting from fouling has instigated extensive investigations of its cause and more particularly of practical methods for its prevention. However, attention has been focused mainly upon the habits, life histories and tolerances to poisonous paints and metals of the macroscopic encroachers such as barnacles, mollusks, tunicates, hydroids, and bryozoans. Surprisingly, little, or no, attention has been devoted to the exact sequence of events, especially during the initial stages, to the relationship of one group of organisms to another, and to the associated microscopic life. It is the purpose of this paper to report the observations which have been made at the Scripps Institution of Oceanography at La Jolla during the last two years on the attachment of bacteria and kindred microörganisms upon submerged surfaces,[1] and to discuss their possible significance with reference to fouling.

The studies of Wilson (1925) at the Scripps Institution on marine algal successions indicated that colonial diatoms were the first

[1] Acknowledgment is here made to Captain A. H. Van Keuren of the Bureau of Construction and Repair of the Navy Department for making available certain invaluable reports and otherwise encouraging the work, and to Professor W. E. Allen of the Scripps Institution for generous assistance.

sessile organisms to appear on submerged plates; but his methods of investigation made no provision for the observation of bacteria. Although he did not apply the procedure to the fouling problem Naumann (1925) reported that, due to their tenacious attachment, excellent preparations of iron bacteria can be prepared by the submergence of glass slides in iron-bearing waters. Both Hentschel (1925) and Thomasson (1925) submerged slides and later examined them microscopically to follow the distribution of diatoms and other minute sessile aquatic organisms, but neither the fouling problem nor bacteria were considered. Using a direct microscopic technique Henrici (1933) concluded from his studies on fresh-water bacteria that "it is quite evident that for the most part water bacteria are not free floating organisms, but grow attached upon submerged surfaces." He found that following the submergence of glass slides in lake water a deposit of bacteria soon becomes apparent and increases progressively until individual cells can be distinguished only with difficulty.

In preliminary observations on the nature and distribution of marine bacteria and their rôle in the fouling of submerged surfaces, ZoBell and Allen (1933), using a procedure somewhat similar to that employed by Henrici, indicated that numerous bacteria soon attach themselves to glass slides submerged in sea water and that bacteria, and to a lesser extent diatoms and actinomyces, usually precede the attachment of barnacles and other fouling organisms. This report is a continuation of those studies.

METHODS OF INVESTIGATION

Standard micro-slides were submerged off the end of the Institution's pier which extends one thousand feet seaward from shore. The slides were tied to a carrier (fig. 1) which consists of a piece of lead about 12 by 4 by 0.25 inches covered with wood, and the whole coated with paraffin. The heavy lead gives anchorage and stability, and the wood and paraffin keep the slides from direct contact with the metal. Grooved wood strips and cord string provide for fastening twelve to sixteen slides on the faces of the carrier. The device was suspended by means of a cotton rope in from six to twelve feet of water, depending upon

the tide. Within these limits the depth of submergence did not influence the results and neither did the tidal phase, the main effect of the latter being merely to change the depth of the water, because the pier virtually extends in the open ocean. Adherent

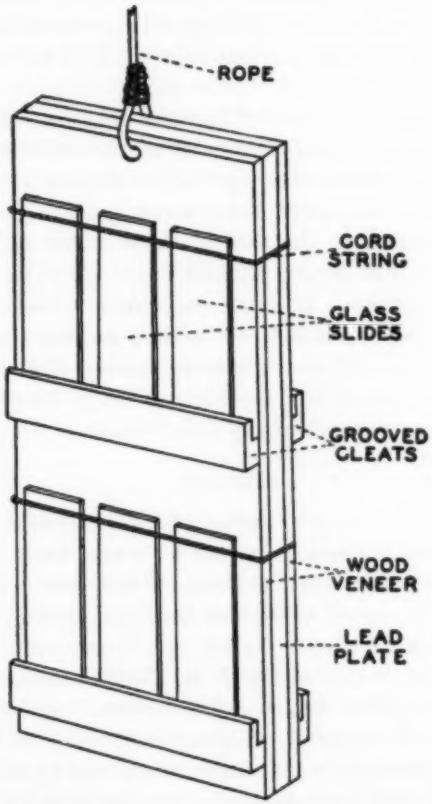

FIG. 1. WOOD COVERED LEAD CARRIER FOR HOLDING SUBMERGED GLASS SLIDES

growths were scraped from the carrier as often as required and the latter was sterilized by dipping in hypochlorite solution. Prior to submergence the slides were cleaned, wrapped in paper, and heat sterilized. Aseptic precautions were exercised in their

manipulation. They were exposed to the sea water for periods varying from a few hours to a few days. They were examined in the fresh condition and also after staining. Loeffler's methylene blue and Hucker's ammonium-oxalate crystal-violet are useful stains; but the most satisfactory results have been achieved with Conn's (1918) rose bengal consisting of 1.0 per cent of the dye and 0.02 per cent anhydrous calcium chloride in 5 per cent aqueous phenol solution.

In order to obtain cultures of bacteria that form films or those which attach to solid surfaces, some of the exposed slides were plated directly in sea-water agar after rinsing them in sterile water to dislodge all except the desired types. This procedure was practicable only in the case of those slides which had been submerged for a few hours, because later there were too many bacteria on the slides. In other experiments bacteria from the adherent films were brushed off with a cotton swab in sterile sea water and appropriate dilutions thereof were plated. The method had no quantitative significance but it furnished a means of obtaining pure cultures of the film-formers.

RESULTS

The majority of the organisms which are found attached to slides submerged in the sea for one to seven days are definitely microscopic in size and most of these are bacteria. Table 1 illustrates the kind of record which was kept and shows the number of bacteria, other microscopic forms, and larger organisms visible to the naked eye, found on the 2- by 1-inch exposed area of the slide after twenty-four hours' submergence. All of the macro-organisms were enumerated, whereas the number of bacteria and other microörganisms per slide were calculated by counting those which appeared in a representative number of fields (magnification 980× and 430×, respectively) and multiplying by a factor. Usually at least fifty fields were scrutinized.

Figure 2 shows graphically the number of bacteria by weekly averages which were found attached to slides which had been submerged for forty-eight hours during the first six months of 1933. There were millions of attached bacteria per 2 square-

inches of slide while concomitant plate counts revealed only hundreds of bacteria per cubic centimeter of water in which the slides were submerged. The lack of relationship between the number of marine bacteria found attached to slides and the number demonstrated by plating procedures is noteworthy.

Controlled laboratory experiments devised to simulate field conditions show that there are several factors besides the number of bacteria present in the sea water which influence the number found attached to glass slides submerged therein. In the first place, not all marine bacteria attach themselves to glass slides even under favorable conditions. This was revealed by testing

TABLE 1

Number of bacteria, other microscopic organisms and macroscopic organisms attached to 2 square inches of micro-slides submerged in the sea for 24 hours

DATE OF SUBMERGENCE	BACTERIA	OTHER MICROSCOPIC ORGANISMS	MACROSCOPIC ORGANISMS
February 7, 1933	2,820,000	3,500	0
February 13, 1933	1,860,000	500	0
February 14, 1933	1,260,000	8,900	0
February 20, 1933	600,000	500	0
February 27, 1933	9,060,000	1,000	0
March 6, 1933	7,800,000	10,300	1
March 9, 1933	6,720,000	12,000	0
March 15, 1933	3,240,000	400	1
March 20, 1933	1,920,000	300	0
March 29, 1933	4,260,000	600	0

73 pure cultures, differing physiologically or morphologically, which had been isolated at random by planting sea water (ZoBell and Feltham, 1934). These were cultivated in bottles of sea-water broth into which sterile glass slides were inserted vertically. After two days' incubation at 25°C. it was found by microscopic examination of the slides that only one-third, or 24 of the 73 cultures, were firmly fixed to the solid surfaces. However, three of the cultures were found attached only to solid surfaces, forming films of micro-colonies on the glass slides and the walls of the bottles, while the broth itself was not perceptibly turbid.

Micro-colonies seldom appear on slides exposed to sea water

for only a few hours; the cells for the most part occur singly or in pairs, or more rarely, in long chains. The paucity of microcolonies is attributed to the lack of available nutrients in sea water, because they appear abundantly on slides in sea water to which nutrients have been added. Under the latter conditions

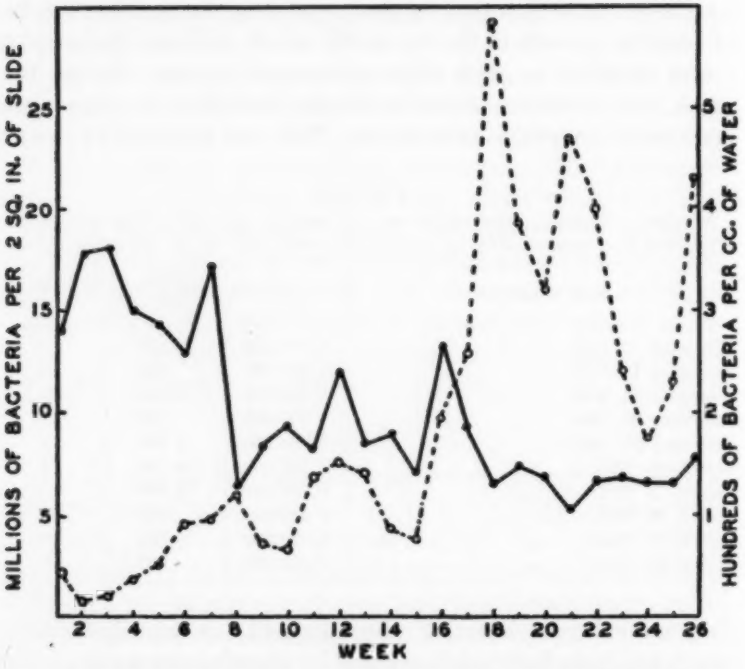

FIG. 2. Broken line represents millions of bacteria found attached to two square inches of submerged slide in forty-eight hours and solid line gives the average plate count per cubic centimeter of sea water for the first twenty-six weeks in 1933.

it is quite evident that the bacteria are multiplying on the surface of the slide and all stages of cell division can be observed. Ordinarily it requires two to four hours for appreciable numbers of bacteria to become attached solidly to glass slides. Firm attachment after coming into contact with the surface is not immediate,

as it seems to take several minutes for the bacteria to cement themselves to the glass. Thus, the submergence of a slide in sea water replete with bacteria, followed by its immediate removal, does not result in the attachment of bacteria, although a few may adhere temporarily. But let the slide remain submerged for an hour or two, and if the bacteria are of the attachment type and are in the logarithmic phase of growth, they will be found profusely, so firmly glued to the slide that running water will not detach them.

Various sizes and shapes of bacteria appear. Ovoid organisms with a diameter of less than 1 micron are most numerous. Such coccobacilli comprise at least 60 per cent of the total number of bacteria. Slender bacilli, 1μ to 2μ in length, are common. Larger rods occur less frequently. True cocci and spirilla are seldom seen. The majority of these organisms possess well-defined capsules which in some cases are twice or three times the size of the enclosed bacterium. Nearly all of the bacteria are Gram-negative.

Assorted filamentous forms occasionally appear. These are of two principal types: (*a*) actinomyces, consisting of small patches of slender mycelial threads which appear both continuous and fragmented, and (*b*) larger, straighter, branched filaments which are probably Chlamydobacter. Leptothrix has been observed.

While it is impossible to identify species of bacteria from a consideration of morphological features only, it is estimated from the diversity of form, size and structure, that no less than forty or fifty species of bacteria are represented regularly, and probably more. Twenty-eight pure cultures have been isolated from twenty-four-hour films for further study. Immediately following isolation the majority of these grow readily in sea-water media, but not in a corresponding nutrient solution prepared with fresh water, thereby indicating their halophilic specificity. Several of these have been completely characterized according to the methods of the Committee (1930) on the Pure Culture Study of Bacteria, and they are found to be new species.

Three representative species which have been encountered frequently attached to submerged surfaces are herewith described.

No. 577. *Achromobacter marinoglutinosus*, n. sp. Short Gram-negative rods 0.7 to 1.0 by 1.8 to 2.4 microns with rounded ends. Methylene blue shows granular structure. Occur singly, in pairs, and in clumps. Encapsulated. Motile by means of polar flagella.

Gelatin stab: Moderate filiform growth with slight napiform liquefaction. No pigment.

Agar slant: Moderate, filiform, flat growth. Butyrous consistency.

Agar colonies: Round with concentric circles and crinkled radial line, 1.5 to 5.0 mm. in diameter. No pigment.

Broth: Moderate clouding, marked ring, adherent film of growth on test-tube wall, and flaky sediment. No growth in milk or on potato.

No indol.

Produces hydrogen sulphide and ammonia from Bacto-tryptone.

Reduces neither nitrate nor nitrite.

Does not ferment glucose, lactose, sucrose or mannitol.

Produces acid but no gas from xylose and dextrin.

Starch hydrolyzed.

Facultative aerobe.

Optimum temperature, 20° to 25°C.

No. 580. *Achromobacter membranoformis*, n. sp. Rods 0.9 to 1.2 by 3.5 to 4.8 microns, occurring singly and in pairs. Encapsulated. Motile by means of lophotrichous flagella.

Gelatin stab: Filiform growth, best at top with slow crateriform liquefaction.

Agar slant: Moderate, beaded, raised growth. Membranous consistency. Becomes browned with age.

Agar colonies: Circular 1.0 to 2.5 mm. with crinkled surface.

Broth: Slight clouding, flocculent sediment, film of growth on walls of test tube.

No growth in milk or on potato.

No indol or hydrogen sulphide.

Reduces neither nitrate nor nitrite.

Produces acid without gas from glucose, sucrose, dextrin, and mannitol. Lactose and xylose not fermented.

No diastatic action.

Aerobic.

Optimum temperature, 20° to 25°C.

No. 588. *Flavobacterium amocontactus*, n. sp. Slender Gram-negative rods, 0.4 to 0.7 by 1.6 to 2.3 microns, with rounded ends. Stain

very lightly. Occur singly or in irregular clumps. Possess well-defined capsules. Actively motile by means of peritrichous flagella.

Gelatin stab: Good filiform growth with rapid saccate liquefaction.

Agar slant: Abundant, filiform, smooth, glistening bright yellow growth having a butyrous consistency. Originally liquefied agar but this property was lost following artificial cultivation.

Agar colonies: Circular 2.0 to 4.0 mm. in diameter, yellow.

Broth: Good growth in sea-water broth with ring at surface, strong clouding and abundant viscid sediment. No odor.

No growth in milk.

No growth on ordinary potato but slight yellow growth on potato dialyzed in sea water.

No indol.

Produces hydrogen sulphide.

Ammonia liberated from peptone.

Reduces both nitrate and nitrite.

Does not ferment glucose, lactose, sucrose, xylose or mannitol.

Starch not attacked.

Facultative aerobe.

Optimum temperature, 18° to 21°C. Optimum reaction, pH. 8.0.

The microscopic organisms other than bacteria which become attached to the submerged slides consist chiefly of diatoms, the common genera being Grammatophora, Navicula, Licmophora, Fragilaria, Striatella, and Nitzschia. Although the diatoms are much less numerous than bacteria, they are always more abundant than the macroscopic organisms. The data summarized in table 2 show that approximately a hundred times as many bacteria as of all other classes of organisms combined were attached to the submerged slide during the primary stages. Furthermore, it is of interest to note that the accumulation of bacteria and, more particularly, of diatoms does not proceed in arithmetical progression with twenty-four-hour intervals; or, in other words, when the number of attached organisms is plotted against time, a parabolic curve rather than a straight line results. This indicates either a multiplication of the attached organisms or a favoring influence of the film-formers upon subsequent attachment. Both factors are probably operative.

As indicated by table 2, very few macroscopic organisms, or

those which could be located without the aid of a lens, were attached to slides which had been submerged for only three days. Their number continued to increase slowly but progressively from the fourth to the seventh day, the longest submergence period considered in these studies. Suctoria and hydroids were most abundant, both appearing with regularity throughout the year but in appreciable numbers only after five days. Cyprid larvae of barnacles were found occasionally from March to August, being common only in the last four months of that period. Most of the larvae were not firmly attached to the slides in seven days and were readily washed off. Bryozoa appeared very rarely. For more information concerning these macroscopic sedentary marine organisms in this vicinity the reader is referred to Coe (1932).

TABLE 2

Average number of different classes of organisms, which were attached to 2 square inches of slide after 24, 48, and 72 hours' submergence during the first six months of 1933

PERIOD OF SUBMERGENCE	BACTERIA	OTHER MICROÖRGANISMS INCLUDING DIATOMS	MACROSCOPIC ORGANISMS
hours			
24	2,023,800	2,560	0.3
48	9,268,200	10,840	1.2
72	24,115,400	28,310	1.9

What is the relation of the primary bacterial film to the attachment of other forms? To elucidate this point further, film-coated glass slides were submerged concurrently with sterile slides as controls. The films were prepared in the laboratory by placing slides in bottles of nutrient broth inoculated with cultures of attachment bacteria until a good film had formed. Following one to five day's submergence in the sea the film-coated and the originally sterile slides were examined for attached organisms. The film-covered slides had a noticeably greater number of attached organisms than did the slides which were originally sterile. Table 3 presents the average results.

Not only do bacteria play an important rôle as primary film-formers, but they are also found in abundance associated with the

growths on fouled surfaces during the later stages of fouling. Several specimens of slimy film were scraped from the hull of the U. S. N. Destroyer LAUB as it was being dry-docked. The vessel had been in the water for thirty-eight months. Direct microscopic analyses by a modified Breed and Brew (1916) method of this material properly diluted revealed that it contained from a few million to several billion bacteria per gram of moist scrapings. From preliminary results which will be reported in greater detail elsewhere it is estimated that, on an average, 8 to 9 per cent by volume of the fouling cumulation on this particular vessel consisted of bacteria. This plethora of bacteria is attributed to the abundance of organic matter on which they feed and to their sedentary properties.

TABLE 3

Average number of microörganisms (excluding bacteria) and macroscopic organisms attached to 2 square inches of sterile micro-slides and to slides coated with a bacterial film under comparable conditions

PERIOD OF SUBMERGENCE	MICROÖRGANISMS		MACROÖRGANISMS	
	Sterile slides	Film-coated slides	Sterile slides	Film-coated slides
hours				
24	15	42	0.3	5.4
48	23	89	1.1	7.2
72	98	276		
120	852	1,257	18.6	43.8

Hilen (1923) reported that the slime which forms on surfaces in ocean water is "composed of a variety of bacteria as well as yeasts and molds." Corroborating these studies, Angst (1923) found that the slime on ships' bottoms is caused to a large extent by bacteria, and, furthermore, he concluded that "the slime bears a direct relation to the appearance of the barnacles."

CONCLUSIONS

Our observations show quite conclusively that bacteria and, to a lesser extent, other microörganisms are the primary film-formers on submerged glass slides, and that such films favor the subsequent attachment of the larger and more inimical fouling

organisms. The film of bacteria may promote the attachment of macroscopic organisms in different ways. They may form a mucilaginous surface to which the fouling organisms in the planktonic or free-swimming stage readily adhere until they can prepare their own holdfast. Again, the bacterial film together with the particulate organic detritus which sticks to it, may furnish the fouling organisms with a suitable source of food during their infancy. Speaking of barnacle larvae in captivity, Visscher (1928a) says, "These organisms have been observed to 'walk' for considerable distances, and have been seen to 'test' various areas for a period of more than an hour before finally attaching." It is possible that at least a part of this delay in attaching may be due to the influence of microörganic films. Furthermore, while it is still a matter of conjecture, it is entirely possible that in the case of submerged surfaces which contain substances poisonous to the fouling organisms, the bacterial film forms a protective coating.

While it remains to be proved that barnacles, bryozoa, hydroids and other macroscopic fouling organisms will not attach without the aid of a primary bacterial or other microörganic film, the foregoing studies suggest that microörganisms merit considerable attention in investigating the exact cause and ultimate prevention of fouling.

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DECOMPOSITION OF SALTS OF ORGANIC ACIDS BY BACTERIA OF THE GENUS SALMONELLA

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In 1924 Brown, Duncan and Henry (1924–5) studied the behavior of various bacteria in 1 per cent peptone broth containing 1 per cent of salts of organic acids, and used a saturated solution of lead acetate to detect decomposition of the salts. Peptone was employed as a source of nitrogen and energy and the salts of organic acids as source of carbon. By the behavior of bacteria investigated they were able to apply the results to the differentiation of bacteria.

Jordan and Harmon (1928) repeated the experiments of Brown and his co-workers, using the same technique. In general their results have confirmed the observations of the English investigators, except in the case of the salts of tartaric acids.

The object of this study was to find what results might be obtained in synthetic media of known chemical constitution, and to investigate the qualitative nutrient requirements when salts of organic acids are utilized as sources of energy and carbon.

In an extensive study of the ability of the various organisms of the Genus Salmonella to decompose various salts of organic acids, it was found that of the salts tested in synthetic media, very few were found to be readily acted upon by these organisms. Therefore only salts of the following open-chain acids need be mentioned:

Dicarboxylic acids
- saturated
  - succinic acid $COOH \cdot CH_2 \cdot CH_2 \cdot COOH$
- unsaturated
  - fumaric acid $COOH \cdot CH \cdot CH \cdot COOH$

Hydroxymonocarboxylic acid
lactic acid $CH_3 \cdot CHOH \cdot COOH$
Hydroxydicarboxylic acid
mucic acid $COOH \cdot (CHOH)_4 \cdot COOH$
Hydroxytricarboxylic acid
citric acid $CH_2 \cdot (COOH) \cdot C(OH) \cdot COOH \cdot CH_2 \cdot COOH$

These salts were used individually in a concentration of 0.5 per cent in various basic media containing brom thymol blue as indicator. Media, purely of synthetic nature such as that formulated by Koser (1923) in his study of the ability of colon-aerogenes organisms to utilize citric acid as a source of carbon, and as formulated by Simmons (1926) in his differentiation of organisms of the typhoid-colon-aerogenes group and for the isolation of certain fungi, were selected as bases of investigation. They had the following compositions:

Liquid (Koser's):

Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.02 per cent
Monobasic ammonium phosphate ($NH_4H_2PO_4$)	0.1 per cent
Dibasic potassium phosphate (K_2HPO_4)	0.1 per cent
Sodium chloride (NaCl)	0.5 per cent
Brom thymol blue (1.6 per cent alcoholic solution)	5 cc. per liter

Solid (Simmons'):

In addition to the liquid medium, Bacto-agar	2.0 per cent

After dissolving the ingredients, each medium was titrated to pH 7.0 (if necessary) with N/1 NaOH and was then tubed in approximately 10 cc. amounts (in case of liquid) and 15 cc. (in case of solid for pouring plates), and was sterilized in the autoclave for fifteen minutes at 15 pounds pressure. Each medium was divided into two series; one was inoculated with a loop from twenty-four hour tryptophane broth cultures, and the other with a straight needle from twenty-four hour beef-extract agar slants. The inoculated tubes and plates were incubated at 37°C. in a well ventilated incubator. Daily observations of growth and changes in reaction were made up to the end of ninety-six hours, which, in the writer's opinion, was considered sufficient.

The synthetic sources of available nitrogen for energy and metabolism were primary and dibasic ammonium phosphates, ammonium chloride, and ammonium sulphate (0.3 per cent). In each

experiment all the aforementioned salts of ammonium were used individually in the media.

Discussions on the use of liquid and solid media. Simmons has observed that at times some confusion may be due to the turbidity produced by bacteria inoculated in synthetic liquid media even when they had not increased in numbers. His observation led to the belief that a solid medium containing an indicator might obviate this disadvantage, since bacterial growth on such a solid medium is always clearly indicated by colony formation, and usually by a striking change in the color of the indicator.

On the other hand, Thompson (1927) felt that the liquid medium is preferable since slight amounts of peptone carried over with the inoculum from the broth or the slants would be less likely to give false results when diluted in a considerable volume of liquid than when smeared on the surface of a solid medium.

To overcome objections in regard to the use of either medium, we have followed both procedures to check the results obtained both from the liquid and the solid media.

The following organisms of the Genus Salmonella studied were:

	Number of strains
Salmonella paratyphi	4
Salmonella Schottmuelleri	5
Salmonella Hirschfeldii	3
Salmonella aertrycke	10
Salmonella enteritidis	6
Salmonella suipestifer	2 (European)
Salmonella suipestifer	2 (American)
Salmonella Morganii	2
Salmonella abortivo-equina	3
Salmonella anatis	2
Salmonella pullorum	3
Salmonella sanguinarum	4

The majority of the strains of organisms employed in this investigation were stock cultures received from various laboratories; the rest have been used to make vaccines and antigens for the agglutination tests. The writer is indebted to Dr. Jordan of Chicago, Dr. Park of New York City, and Dr. Wadsworth of New York State for the majority of strains of bacteria, and to Dr. Topley of London for the European strains of *Salmonella suipestifer* and other cultures.

DISCUSSION

Investigation into the nutrient requirements of bacteria of the Genus Salmonella for nitrogen available from inorganic sources to decompose salts of organic acids for source of carbon and energy showed no differences among different species. The majority of species thrive well in a medium containing N in the form of either primary or dibasic ammonium phosphate, ammonium chloride, or ammonium sulphate, P as phosphate, and C in the form of organic acid. Although Mg is not necessary for bacterial metabolism, it has been observed that more abundant growth is obtained when Mg is present in the medium than when it is not, apparently due to the necessity of the presence of bivalent cations with the monovalent cations. No attempt was made to investigate the necessity of K, S, or other cations for bacterial metabolism.

Results obtained from synthetic media may be conveniently summarized with short discussions on each type.

Salmonella paratyphi, *Salmonella Morganii*, *Salmonella abortivo-equina*, *Salmonella pullorum* and *Salmonella sanguinarum* repeatedly failed to grow in any of the salt media, even upon prolonged incubation.

According to Bergey, *Salmonella Hirschfeldii* is considered morphologically and culturally similar to *Salmonella enteritidis* but differs in its serologic reactions. In synthetic media, it may readily be distinguished from *Salmonella enteritidis* and also from *Salmonella Schottmuelleri*, *Salmonella aertrycke*, *Salmonella anatis* and *Salmonella suipestifer* (European) by its inability to decompose salts of succinic and fumaric acids. It regularly decomposes salts of lactic and citric acids with the production of alkaline compounds. There are no differences between the strains of *Salmonella Hirschfeldii* and those of *Salmonella suipestifer* (American).

The American strains of *Salmonella suipestifer* differ from the European strains by their failure to decompose succinic and fumaric acids. They are both, however, able to decompose lactic and citric acids, and both fail to decompose, to any extent, mucic acid.

Salmonella Schottmuelleri, *Salmonella aertrycke* and, *Salmonella*

anatis exhibited considerable regularity in attacking all of the five salts mentioned. *Salmonella enteritidis* is irregular in its attack upon mucic acid. These relations toward mucic acid may be due to the ability or inability of certain strains to attack the compound rather than to the specific adaptability of the species to attack it.

It is interesting to note that all of the strains of *Salmonella Schottmuelleri, Salmonella aertrycke, Salmonella anatis* and some strains of *Salmonella enteritidis* decompose mucic acid, in synthetic liquid media, with the production of acidity, in the first hours of incubation, and, after incubation at 37°C. for forty-eight hours, the pH of the medium reverts to neutrality. With further incubation, the medium is turned deep Prussian blue. On solid medium, these types of organisms decompose mucic acid with the production of alkalinity—unlike the reaction observed in the liquid media.

Brown and his co-workers claim that succinic acid is apparently not readily decomposed by any member of the Salmonella group or allied types. Yet, they observed that when the cultures in fumaric acid were incubated for four days, a second phase of fermentation occurred with the apparent decomposition of fumaric acid into succinic acid, carbonates and other volatile acids. We have found that all of the strains of *Salmonella Schottmuelleri, Salmonella aertrycke, Salmonella enteritidis, Salmonella anatis* and *Salmonella suipestifer* (European) that decompose fumaric acid also attack succinic acid.

The action of each type of organisms on the five salts of organic acids is tabulated, along with the observations made by Brown and his co-workers on the distribution of fermentative activity in citric, fumaric and mucic acids with peptone as indicated in table 1.

It will be noted that the results obtained from the synthetic media, using either primary or secondary ammonium phosphate, ammonium chloride, or ammonium sulphate as source of nitrogen, are in perfect agreement with those obtained from peptone broth by Brown and his co-workers, except in one case, that of *Salmonella enteritidis* in mucic acid. Unfortunately, they did not report the result of *Salmonella Schottmuelleri* with fumaric acid,

although the Medical Research Council (1929) reported the reaction as negative.

Detection of decomposition of organic acids by bacteria in peptone broth by the addition of lead acetate solution involves a considerable time factor. In the application of this test a floccular precipitate indicates that the salt has not been decomposed. The decomposition of the organic acid is indicated by the absence of floccular precipitate. It is believed that an equally

TABLE 1

	RESULTS AFTER BROWN			RESULTS OBTAINED IN SYNTHETIC MEDIA				
	Fumarate	Mucate	Citrate	Succinate	Fumarate	Lactate	Mucate	Citrate
S. paratyphi	−	−	−	−	−	−	−	−
S. Schottmuelleri		+	+	+	+	+	+	+
S. Hirschfeldii	−	−	+	−	−	+	−	+
S. aertrycke	+	+	+	+	+	+	+	+
S. enteritidis	+	+	+	+	+	+	±	+
S. suipestifer (European)	+	−	+	+	+	+	−	+
S. suipestifer (American)				−	−	+	−	+
S. Morganii				−	−	−	−	−
S. anatis				+	+	+	+	+
S. pullorum				−	−	−	−	−
S. sanguinarum				−	−	−	−	−
S. abortivo-equina				−	−	−	−	−

+, decomposition with production of alkaline compounds and other acids; −, no decomposition at the end of ninety-six hours' incubation; ±, irregularity of *S. enteritidis* to attack mucate.

certain result can be obtained with a considerable saving of time by simple inspection as the cultures unable to decompose salts of organic acids remain clear and the indicator unchanged in contrast to the turbidity and the change of indicator seen in the cultures able to decompose salts.

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FURTHER STUDIES ON SPONTANEOUS VARIATIONS OF TORULA PULCHERRIMA

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In a previous paper (Punkari and Henrici, 1933) we have reported upon spontaneous variations in a chromogenic asporogenous yeast, *Torula pulcherrima*. Originally described as a colorless Torula with a red soluble pigment, this yeast was found, in giant colonies, to give rise to variants which were colored maroon and others which were without pigment; and also to rough variants which showed rudimentary mycelium. These appeared as sectors or as secondary colonies in old giant colonies.

These observations have been continued through six further "generations." The observations reported in this paper deal with the offspring from one of the colonies, Dlcz, occurring in the fourth "generation" of the studies reported in the previous paper. Ths history of this colony has been given in the former paper, and its genealogy is recapitulated in diagrammatic form in figure 1. The colony was white, with a rough red center. From the white margin of this colony there was obtained a colony of the same type (I). This colony I has been the starting point for a further series of studies.

In our former work, single cell isolations were made between transfers, i.e. each giant colony was started from a single cell. This has not been continued because we believe that the purity of the line is established beyond any question of doubt; because the single cell isolation involves much more time; and more especially because it introduces an uncontrollable element of selection which undoubtedly plays some part in determining the variations observed. Giant colonies were obtained by touching a straight inoculating wire to some particular part of the colony,

then transferring directly to the surface of the agar in a bottle. With this exception, the technique followed was the same as that employed in our earlier studies.

In the following description of the colonies, two terms which were not used in our previous paper require definition. "Rays" may be looked upon as the converse of sectors. Starting from the central portion of the colony, they overgrow its surface towards the periphery, tapering as they do so. "Craters" are, as

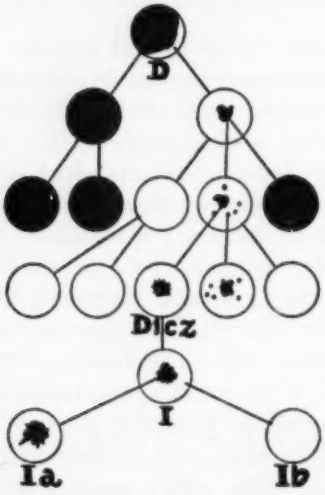

FIG. 1. DIAGRAM SHOWING THE ORIGIN OF COLONIES Ia AND Ib FROM COLONY D, WHICH WAS DERIVED BY SINGLE CELL ISOLATION FROM A STOCK CULTURE

Black indicates red pigment. No attempt has been made to show roughness and smoothness.

their name implies, crater-like depressions on the surface of the colony, appearing as though a gas bubble had burst at this point. At first we believed that these were caused by gas bubbles, and carefully compared a number of cultures from colonies with craters with cultures from smooth colonies for rate and amount of gas formation from glucose, but could detect no significant difference. On the other hand, microscopic examination of colonies with craters always showed the presence of mycelium, which was

not found in smooth colonies. Craters have always been observed in white colonies, and characteristic "rough" white colonies have never been seen. In our previous paper we pointed out that "rough" red colonies always showed mycelium on microscopic examination. We therefore conclude that craters are the form of "roughness" peculiar to the white variants of this yeast.

From the parent colony I, two daughter colonies, Ia and Ib, were obtained. Colonies of the next generation are designated Ia1, Ia2, etc.; their offspring were labelled Ia1a, Ia1b, etc. By this system the origin of any colony may be determined from its label. Below is a description of the various colonies.

I. From the white margin of Dlcz. Smooth white with a rough red center.

Ia. From the red center of I. Smooth white, with red center and red rays extending toward the periphery.

Ib. From the white margin of I. Smooth white, with craters near the center.

Ia1. From a red ray of Ia. Smooth red, with two small white sectors.

Ia2. From the red center of Ia. Dark red with white margin.

Ia3. From the white margin of Ia. Smooth white.

Ib1. From the white margin of Ib. Smooth white.

Ib2. From the white center of Ib. Smooth white, with a few craters.

Ia1a. From the white sector of Ia1. Smooth white.

Ia1b. From the red center of Ia1. Smooth red, *with red soluble pigment in the medium.*

Ia2a. From the white margin of Ia2. Smooth white, with a few craters.

Ia2b. From a red ray of Ia2. Smooth light red with red soluble pigment in the medium.

Ia2c. From the red center of Ia2. Smooth red with white sectors; red soluble pigment in the medium.

Ia3a. From the white center of Ia3. Smooth white.

Ia3b. From the white margin of Ia3. Smooth white.

Ib1a. From the white center of Ib1. Smooth white with a few craters.

Ib1b. From the white margin of Ib1. Smooth white, with a few craters, and red secondary growth in the center.

Ib2a. From the white margin of Ib2. Smooth white with a few craters.

Ib2b. From the white center of Ib2. Smooth white with craters.

Ia1a1. From the white center of Ia1a. Smooth white.

Ia1a2. From the white margin of Ia1a. Smooth white with craters.

Ia1b1. From the red margin of Ia1b. Smooth red; red soluble pigment in medium.

Ia1b2. From the red center of Ia1b. Smooth red with light red margin: red soluble pigment in the medium.

Ia2a1. From the white center of Ia2a. White, with many craters.

Ia2a2. From the white margin of Ia2a. White, with many craters.

Ia2b1. From the red margin of Ia2b. Slightly rough, light red, with red soluble pigment in the medium.

Ia2b2. From the red center of Ia2b. Smooth red with light red margin. White secondary papillae.

Ia2c1. From white sector of Ia2c. Smooth white.

Ia2c2. From red center of Ia2c. Smooth white with light red secondary growth and rays.

Ia3a1. From white center of Ia3a. Smooth white with craters.

Ia3b1. From white center of Ia3b. Smooth white with craters.

Ia3b2. From white margin of Ia3b. Smooth white with two white sectors showing craters.

Ib1a1. From the white center of Ib1a. Smooth white with craters.

Ib1a2. From the white margin of Ib1a. Smooth white with craters.

Ib1a3. From the white craters of Ib1a. Smooth white with many craters.

Ib1b2. From the red secondary growth of Ib1b. Smooth white with red secondary growth and a few craters.

Ib2a1. From the white center of Ib2a. Smooth white with craters.

Ib2a2. From the white margin of Ib2a. Smooth white with a few craters.

Ib2a3. From the white craters of Ib2a. Smooth white with craters.

Ib2b1. From the white center of Ib2b. Smooth white with a few craters, and two white sectors showing many craters.

Ib2b2. From the white margin of Ib2b. Smooth white with few craters.

Ia1a1a. From the white center of Ia1a1. Smooth white with craters.

Ia1a2a. From the white center of Ia1a2. Smooth white with few craters.

Ia1b1a. From the red center of Ia1b1. Smooth light red with red soluble pigment in the medium.

Ia1b1b. From the red margin of Ia1b1. Smooth red with red soluble pigment in the medium.

Ia1b2a. From the red center of Ia1b2. Smooth red with slight roughness in the center; red soluble pigment in the medium.

Ia1b2b. From light red sector of Ia1b2. Smooth light red, margin dark red; red soluble pigment in the medium.

Ia1b2c. From light red margin of Ia1b2. White with craters, red rough center, and smooth white sectors.

Ia2a1a. From the white center of Ia2a1. Smooth white.

Ia2a1b. From the white margin of Ia2a1. Smooth white.

Ia2a2a. From the white center of Ia2a2. Smooth white with few craters.

Ia2a2b. From the white margin of Ia2a2. Smooth white with large craters.

Ia2b1a. From the red center of Ia2b1. Smooth red with rough center and smooth white peripheral portion.

Ia2b1b. From the light red margin of Ia2b1. Smooth red with rough center and white sectors.

Ia2b1c. From the smooth red portion of Ia2b1. Smooth red with rough center.

Ia2b2a. From the red center of Ia2b2. Smooth light red with red soluble pigment in the medium.

Ia2b2b. From the light red margin of Ia2b2. Smooth red with rough center and white sector.

Ia2b2c. From the secondary papillae of Ia2b2. Smooth white.

Ia2c1a. From the white center of Ia2c1. Smooth white.

Ia2c2a. From the red center of Ia2c2. Smooth red, with red soluble pigment.

Ia2c2b. From the red ray of Ia2c2. White with craters and red rough center; no soluble pigment.

Ia2c2c. From the white margin of Ia2ca. Smooth white.

Ia2a1a. From the white center of Ia3a1. Smooth white with very few craters.

Ia3a1b. From the white margin of Ia3a1. White with many craters.

Ia3a1c. From the white sector of Ia3a1. Smooth white with craters.

Ia3b1a. From the white center of Ia3b1. Smooth white.

Ia3b1b. From the white margin of Ia3b1. Smooth white with craters.

Ia3b1c. From the white sector of Ia3b1. Smooth white with craters.

Ia3b2a. From the white centers of Ia3b2. Smooth white with craters.

Ia3b2b. From the white margin of Ia3b2. Smooth white with craters.

Ia3b2c. From the white sector of Ia3b2. Smooth white with craters.

Ib1a1a. From the white center of Ib1a1. Smooth white.

Ib1a1b. From the white margin of Ib1a1. Smooth white.

Ib1a2a. From the white center of Ib1a2. Smooth white with few craters.

Ib1a3a. From the white center of Ib1a3. Smooth white.

Ib1a3b. From the white margin of Ib1a3. Smooth white.

Ib1a3c. From the smooth white portion of Ib1a3. Smooth white.

Ib1b2a. From the red rough center of Ib1b2. Smooth red with red rough center, white sector and three light red sectors.

Ib1b2b. From the white margin of Ib1b2. Smooth white.

Ib1b2c. From the white craters of Ib1b2. Smooth white with craters.

Ib2a1b. From the white margin of Ib2a1. Smooth white.

Ib2a1c. From the white sector of Ib2a1. Smooth white.

Ib2a2a. From the white center of Ib2a2. Smooth white.

Ib2a2b. From the white margin of Ib2a2. Smooth white with few large craters.

Ib2a3a. From the white center of Ib2a3. Smooth white.

Ib2b1a. From the white center of Ib2b1. Smooth white.

Ib2b1b. From the white margin of Ib2b1. Smooth white with few small craters.

Ib2b1c. From the white sector of Ib2b1. Smooth white.

Ib2b2a. From the white center of Ib2ba. Smooth white with craters.

Ib2b2b. From the white margin of Ib2b2. White with many craters.

Ib2b2c. From the white craters of Ib2b2. Smooth white with craters.

Torula pulcherrima was originally described as a white yeast with a maroon-colored soluble pigment, and the strain which we have studied fitted this description when first received. However, for some years before these studies were undertaken, it had failed to produce any soluble pigment; instead, the growth itself was deeply colored, without any trace of color in the agar. It was interesting to note the sudden reappearance of soluble pig-

ment in colonies developing from colony Ia. In nearly all of the offspring from the red center of this colony, the agar has been colored as well as the growth itself.

The genealogies of these cultures are presented in graphic form in figures 1, 2, and 3. Figure 1 shows the origin of colonies Ia and Ib from colony D of our previous paper; figure 2 shows the offspring of Ia; and figure 3 the offspring of Ib. In these diagrams no attempt has been made to indicate roughness and

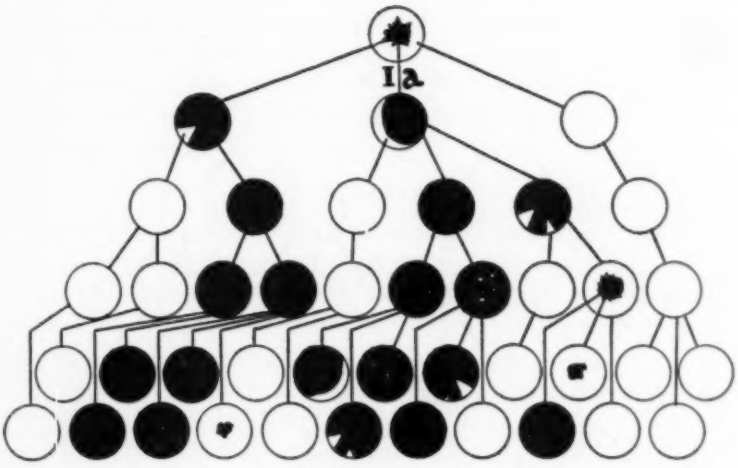

FIG. 2. DIAGRAM SHOWING THE OFFSPRING OF COLONY Ia, WHICH WAS DERIVED FROM THE RED CENTER OF COLONY I

Note that the red portion gave red daughter colonies, which however continue to split off white variants.

smoothness, nor presence and absence of soluble pigment. For lack of space, not all of the colonies of the final generation have been included.

A study of these diagrams brings out two important features:

First, red is much less stable than white. It has been possible to maintain pure reds through three generations in two lines, by selective subculturing. But white sectors appear so frequently in red colonies that we believe it will be impossible, under the

conditions of this experiment, to maintain a pure red line indefinitely. Although a number of subcultures from white sectors have yielded white colonies which after a time developed red centers or papillae, in only one instance has the *offspring* of a colony which remained pure white during observation, yielded a colony which developed red pigment under observation. In this respect, namely stability of the variant and instability of the parent type, the "dissociation" of this yeast is similar to that of

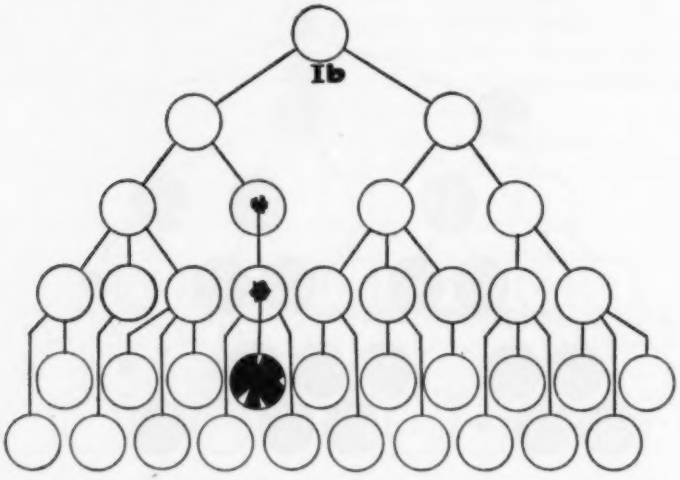

FIG. 3. DIAGRAM SHOWING THE OFFSPRING OF COLONY Ib, WHICH WAS DERIVED FROM THE WHITE PERIPHERY OF COLONY I

Note that, excepting one line, all of the daughter colonies have been pure white

numerous bacteria. One of us (Henrici, 1928, 1934) has discussed this phenomenon, and has pointed out that it cannot be made to fit a Mendelian formula, and that therefore the observed variations cannot be attributed to sexual reproduction.

Secondly, it will be noted that wherever red has appeared in a white colony, both in the present series and in those previously reported, with one exception it has been in the form of a central growth or as secondary papillae; colony *Alcy* in the previous

series was described as smooth white with a red sector. Conversely, with one exception, white has rarely appeared in red colonies as sectors; in colony *Ia2b2* white papillae appeared on a red ground, and a subculture of one of these papillae yielded a pure white colony. In general, then, it would appear that the transformation from red to white occurs in the young growing cells at the periphery of the colony, giving rise to sectors, and may possibly be due to some accident in nuclear division. On the other hand, the reappearance of red in the white variants occurs in the oldest part of the colony, and is somehow related with the rejuvenescence that gives rise to central overgrowths, and secondary papillae. The significance of this observation is quite obscure, but it may prove a fruitful lead in further studies of microbic variation.

SUMMARY

Variations in a chromogenic asporogenous yeast have been studied through six further giant colony generations.

New variants observed are colonies with red soluble pigment, and white colonies with "craters," which are looked upon as rough types.

White variants have been found to be much more stable than the red parent type.

It has been noted in general that white variants arise from red in the actively growing cells at the periphery of the colony, whereas red variants arise from white in the aged cells at the center of the colony.

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THE IDENTIFICATION OF VON HIBLER'S "BACILLUS VI" AS BACILLUS CARNIS (KLEIN)

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Following Pasteur and Joubert's (1877) discovery of "*vibrion septique*" in the putrid blood of a cow affected with septicemia, Koch (1881) demonstrated that laboratory animals inoculated with heated suspensions of garden soil often died with symptoms and lesions of malignant oedema similar to those produced by the "*vibrion septique.*" The still unended controversy over the supposed identity of Pasteur's "*vibrion septique*" and Koch's "*Oedembacillus*" is well known through the more or less futile efforts of Weinberg and Séguin (1917), Heller (1920), Ford (1927), and others, to rationalize the taxonomic and nomenclatural confusion which originated in the above experiment.

We shall not attempt here to unravel the knotted and twisted threads of that tangled skein; we only suggest that the principal merit of these pioneering discoveries lay in their emphasis upon experimental inoculation of animals as a technical point of departure in the solution of the problems of wound infections and in the discovery of new pathogenic bacteria.

In 1899, and again in 1905, von Hibler described the highly pathogenic "*Bacillus der Art V*" which he had recovered from the subcutaneous tissues of a rabbit, dead after inoculation with garden soil. "*Bacillus V*" was motile and Gram-positive, formed spores, and showed no action in milk, gelatin, or brain medium, other than the production of gas. Although in its failure to change milk and gelatin it resembled Klein's "Neuen Bacillus des malignen Oedems" (1891) and Sanfelice's "*Bacillus pseudo-*

oedematis maligni" (1891) (1893), it differed in heat resistance due to its spores, in Gram staining reaction, and in apparent failure to grow aerobically.

In 1908 von Hibler changed the number from V to VI, and in 1912 briefly distinguished the organism from the "*Rauschbrand bacillus*" by its small size and cultural properties. Von Werdt (1912) stated that von Hibler's "*Bacillus VI*" had been observed in human infections, but we have been unable to find other references to such infections, and they must be very uncommon, because in all of the numerous studies of war wounds of the past two decades this organism was apparently not encountered. Neither did Zeissler and Raszfeld (1928) find it in their careful studies of nearly 200 samples of soil from the battle fields of Europe.

Yet there are several incidental references to "*Bacillus VI*" in papers dealing primarily with war wound infections. Thus, Weinberg and Séguin (1917) pointed out the resemblance of "*Bacillus VI*" to von Hibler's "*Bacillus VII*" and to their own *B. fallax*. We shall show, however, that *B. fallax* is a distinct species.

Heller (1922) made "*Bacillus VI*" the type species, "*H. sextus*," of a new genus "*Hiblerillus*," but we see no reason for the genus "*Hiblerillus*," and, while we were at first strongly inclined to accept the specific name "*sextus*," we shall later show that "*sextus*" is invalid on grounds of priority.

Zeissler (1922), (1923a), (1923b), confirmed von Hibler's description of "*Bacillus VI*" in a study of the original strain which he inherited from the old von Hibler collection in Innsbruck.

Lehmann and Neumann (1927) described "*Bacillus VI*" under the specific name "*Bacillus lactiparcus* (*v. Hibler*) (*Lehmann u. Süssmann*)." We were unable to locate the original description by Lehmann and Süssmann, but this name also is invalid, as we shall show. Lehmann and Neumann also mentioned that a strain of "*B. enteritidis sporogenes*" sent by Klein to von Hibler and designated by him as "*Art IV*" was shown by Zeissler to be an impure culture of a second strain of "*Bacillus VI*," which

bears an important relation to our identification of *"Bacillus VI"* as *B. carnis* (Klein).

Zeissler and Raszfeld (1929) recorded the versatile fermentative reactions of these two strains of *"Bacillus VI"* along with those of other anaerobic bacilli, but the most complete description of *"Bacillus VI"* is that of Zeissler (1930), who added three new properties, first, its delicate aerobic growth on glucose blood agar plates, second, its production of a soluble exotoxin, and third, the production of specific agglutinins in immunized animals, all of which we have confirmed and extended. A preliminary report of our observations was published by Duffett (1933).

PERSONAL OBSERVATIONS

Strain 2994 was secured in April 1929 from Dr. Zeissler through Dr. J. P. Scott of Manhattan, Kansas.

Considerable work was done with this culture before we discovered a slowly growing non-pathogenic contaminant closely resembling *Corynebacterium lymphophilum (Torrey) emend Bergey 1930.* We attempted to exclude this contaminant by boiling cultures in deep brain medium, and by picking apparently well-separated colonies from deep agar in Burri tubes and from blood agar slants under alkaline pyrogallol only to have it reappear again and again in subcultures.

Finally recognizing that both *"Bacillus VI"* and the contaminant would develop slowly upon aerobic blood agar plates, we were able, by picking distinctive colonies, to isolate each in unquestioned purity. This led us to secure duplicate cultures both from Dr. Scott and from Dr. Zeissler for comparison and eventually, through the study of Klein's work, which was suggested by a consideration of Zeissler's second strain (our No. 6594), to recognize the probably identity of *"Bacillus VI"* with Klein's *Bacillus carnis.*

In our earlier and apparently futile attempts to secure a pure culture of *"Bacillus VI"* we considered the possibility that we were dealing with a case of microbic dissociation, but the distinctive morphologic, culture, and serologic, properties of the pure cultures finally isolated dispelled all such suspicions.

The following additional cultures were investigated:

Strain 6519, received March 3, 1933 from Dr. J. P. Scott, as a duplicate of Strain 2994.

Strain 6593, received directly from Dr. Zeissler, April 4, 1933, as a dried spore preparation of the original "*Bacillus VI*" from the old von Hibler collection at Innsbruck.

Strain 6594, also received directly from Dr. Zeissler April 4, 1933, as a dried spore preparation from his second strain of "*Bacillus VI*" which he had isolated from a culture of "*Bacillus enteritidis sporogenes*" (*Klein*) found in the old von Hibler collection at Innsbruck.

No impurity was found in the last three strains and their morphological, cultural, and serological properties were proved to be identical with those of our purified strain 2994.

Morphology and staining properties

In young cultures the bacilli appeared, as described by von Hibler (1908), singly or in pairs, rarely in short chains of 3 or 4 cells, Gram-positive, and solidly stained. In older cultures, especially on aerobic media, Gram-negative, swollen, pleomorphic, and granular forms appeared.

The bacilli measured 0.5 to 0.7 micron in width by 1.5 to 4.5 microns in length. The width was more constant than the length, the former averaging 0.6 micron the latter 2.6 microns. Our figures coincide with those of Zeissler (1930), but exceed those of von Hibler (1908) who found that the rods measured 0.32 to 0.64 micron in width by 1.2 to 3.4 microns in length, "*Bacillus VI*" being the smallest of the bacilli which he described.

Spores

Mediums poor in fermentable carbohydrates, such as blood agar slants under alkaline pyrogallol, 2 per cent peptone nutrient broth in constricted tubes, and deep brain, yielded numerous spores in forty-eight hours at 37°C.; as in the case of *Bacillus histolyticus* and *Bacillus tertius*, which also grow aerobically, spores rarely appeared in aerobic cultures on solid media.

After continued cultivation and purification, this property of

active sporulation was apparently lost, as indicated by failure to find spores microscopically and failure of fifteen-day-old deep brain cultures to stand boiling for one minute. However, after such cultures were grown in glucose broth, injected subcutaneously into guinea pigs, and subsequently obtained from the heart's blood of the dead animals, they showed many spores when grown on anaerobic blood agar for forty-eight hours. Von Hibler (1908) described and illustrated the spores as long, ellipsoidal, and terminal. We found them to be generally subterminal, rarely central or terminal, always elongate, and only a little thicker than the rods. Orgonts and spores in young rods, as indicated by the ability of the rods to hold the Gram-positive stain, appeared slightly subterminal. Spores in older rods which failed to hold the Gram stain were sometimes apparently terminal. We interpret this as due to the incorporation of the remaining portion of vegetative protoplasm in the proximal end of the rod very early as the spore grows equally in both directions. Essentially, then, the spores are subterminal in position. Figure 1, plate 1, shows both the spores and vegetative rods of strain 2994.

Von Hibler (1908) found that the spores resisted boiling for five, but not for six minutes in brain broth and other mediums. We found that they resisted boiling (94°C.) for three, but not for four minutes in several tests with forty-eight-hour deep brain and anaerobic blood agar cultures.

The spores were quite resistant to drying, as was shown by the vigorous growth overnight in brain medium from the dried spore preparations received from Zeissler, and by the rapid resuscitation upon incubation in glucose broth of old dried brain cultures.

Motility

The bacilli were sluggishly motile in cover-slip preparations made from cultures in deep brain medium, blood agar slants under alkaline pyrogallol, peptone broth, glucose broth, gelatin, and exudates from guinea pigs dead after inoculation.

Figure 2, plate 1, was made from a slide stained by Bailey's method (1930) to show the flagella, which, as noted by both von Hibler and Zeissler, are peritrichally disposed.

Cultural properties

"Bacillus VI" was described by von Hibler as an obligate anaerobe. We found that it grew readily in the various mediums and devices used in this laboratory in anaerobic work (Hall, 1932) both at 37°C. and at room temperature. We also found that growth could be secured aerobically.

Plain agar. Minute, transparent, dew-like colonies appeared upon agar slants under alkaline pyrogallol after forty-eight hours incubation. These slowly increased in size, becoming flat and lobate.

The early check tests for aerobic growth were made on plain 2 per cent nutrient agar plates. If any growth occurred on these plates it was so delicate that we overlooked it. Later we occasionally detected a delicate surface growth on aerobic agar slants about the second or third day.

After the contaminant in strain 2994 was found, aerobic check cultures were made on blood agar plates, on which *"Bacillus VI"* grew, though much more delicately and more slowly than on anaerobic mediums. We later found that Zeissler had already (1930) made a similar observation on glucose blood agar. All other authors have described *"Bacillus VI"* as obligately anaerobic, having apparently, with the exception of von Hibler, made their descriptions solely from the literature. It follows, therefore, from Zeissler's observations and our own that *"Bacillus VI"* is a micro-aerophilic, not an obligately anaerobic bacillus.

Blood agar. Under alkaline pyrogallol, minute transparent dew-like colonies appeared on slants in twenty-four hours at 37°C. There was no hemolysis at that time. At forty-eight hours the colonies appeared flatter, lobate, and larger in diameter, often attaining 1 to 1.5 mm., with slight but never marked hemolysis. Later they often became confluent.

Aerobic blood agar plates generally showed barely visible nonhemolytic pin point colonies in twenty-four hours. At forty-eight hours the colonies were easily discernible as minute, lobate, slightly spreading, transparent discs, surrounded by diffuse zones of hemolysis. Figure 3, plate 1, shows a section of such a plate.

At seventy-two hours some of the colonies which were well separated became opaque and showed a tendency to round up into pearly beads; others, adjacent, remained flat like those which were more crowded. These differences are not to be interpreted as due to "mutation" or "dissociation," because the same differences appeared in the colonies developing on new plates made from each "type."

Glucose blood agar. Lehmann and Neumann (1927), in describing "*Bacillus lactiparcus*" recorded that there was no hemolysis, apparently following Zeissler (1923a) who described the colonies on glucose blood agar plates as differing from his non-hemolytic "Wuchsform V" only in having notched edges. Some of Zeissler's colonies also resembled those of his non-hemolytic "Wuchsform IIC."

Suspecting Zeissler's failure to observe hemolysis to be due to his use of glucose blood agar, we tried this also, with the curious result that in some instances there was less active growth than on plain agar, and no hemolysis, while in others growth was accelerated with marked hemolysis. We have not succeeded in determining the factor or factors responsible for these variable results but we are sure that the mediums were responsible, because all strains behaved alike on each lot.

Minute dew-like colonies generally appeared on glucose blood agar slants under alkaline pyrogallol after twenty-four hours incubation at 37°C. There was usually no hemolysis until forty-eight hours, but at that time it was quite distinct, each colony being surrounded by a clear-cut hemolytic areola 1 mm. broad which increased daily. Where the colonies were well separated, they became opaque, pearly, yellowish, and convex.

Aerobically, under favorable conditions, the results at twenty-four hours were like those with plain blood agar. At forty-eight hours, each colony was surrounded by a sharply defined hemolytic zone 1 mm. in width, and outside this a narrow green ring. The green ring disappeared on the following day, while the hemolytic zone increased diffusely to a diameter of 8 mm. on the fourth day. Where the colonies were confluent or crowded they remained flat and lobate, but where well separated they became

convex and progressively pearly white, yellowish, orange, and brown, with colorless flat lobate outgrowths. The convex portion of such colonies sometimes attained a diameter of 0.5 mm., while the flat portion often reached 2 mm.

Chocolate agar. Very minute dew-like colonies appeared both aerobically and anaerobically within twenty-four hours. At forty-eight hours they became flat, and remained almost invisible on further incubation. There was no discoloration of the medium to suggest the production of peroxidase.

Löffler's coagulated blood serum. Under alkaline pyrogallol transparent dew-like colonies appeared in about twenty-four hours. After forty-eight hours the colonies were semi-transparent, lobate, and flat, with slightly raised centers. There was no liquefaction even after sixty days. The water of condensation gave an acid reaction to brom-thymol-blue.

Aerobically the growth was similar but appeared more slowly.

Petroff's gentian-violet egg medium. Dew-like colonies appeared anaerobically after twenty-four hours incubation. After forty-eight hours the colonies were larger, lobate, and flat, with slightly raised centers. The medium adjacent to the colonies became distinctly yellow in color after four days, owing to the destruction of the dye, as observed by Hall and Scott (1927) and by Hall, Rymer, and Jungherr (1929) for *Bacillus Sordellii.*

Aerobic colonies took up the dye, but the dye was not destroyed.

Deep brain medium. With iron wire, vigorous production of gas appeared upon twenty-four hours incubation. After two weeks there was slight discoloration of the surface of the medium due perhaps to oxidation, but there was no true blackening of the medium nor tyrosine production upon incubation at 37°C. for sixty days or in one year at room temperature. The German investigators also found that *"Bacillus VI"* did not blacken brain medium. It seems clear, therefore, that little, if any, hydrogen sulfide is formed.

Deep agar. Meat infusion 1 per cent agar with 2 per cent peptone was used in Burri tubes. After twenty-four hours incubation, minute colonies and often gas appeared. Growth extended

to about 1.5 cm. from the ends of the tubes. The use of agar containing one part methylene blue per 100,000 as a criterion of anaerobiosis gave results identical with those secured in agar without the dye; there was no inhibition of growth except near the colored ends of the agar where oxygen was reabsorbed. *It is a curious and as yet unexplained fact that all three of the microaerophilic bacilli, B. tertius, B. histolyticus, and "Bacillus VI," are inhibited in the aerobic layers of deep agar, yet all grow readily on the surface of slants and plates.* After forty-eight hours incubation, the deep lenticular colonies of *"Bacillus VI"* began to show slight protuberances, which later became arborizing processes. With medium or heavy inoculations, gas was always produced during forty-eight hours incubation. Figure 4, plate 1, shows a thirty-seven-day-old colony from a deep agar culture. This colony, being well isolated, attained a diameter of about 4 mm.

Milk. Considerable gas was produced in whole milk in constricted tubes in twenty-four to forty-eight hours, but there was no coagulation or other visible change in the milk even after thirty days incubation. Some milk cultures were kept until practically dry without coagulation. Von Hibler also found that the organism failed to coagulate milk. In his early papers, Zeissler (1922), 1923a), (1923b) reported the same observation. Later, however, Zeissler (1928), (1930), and Zeissler and Raszfeld (1929) reported that milk was coagulated, and that the coagulum shrank to about half the original volume of the milk, but was not liquefied.

Since the contaminant recovered from our culture 2994 produced a reaction in milk similar to that which Zeissler described, we suspected that Zeissler's last observations might have been based upon a culture containing this contaminant. However, the cultures subsequently obtained from Zeissler, failed to coagulate milk and showed no evidence of impurity.

Gelatin. Turbidity and gas appeared in gelatin in constricted tubes with marble seals in twenty-four hours, but there was no liquefaction or darkening of the gelatin even after thirty days incubation at 37°C.; the cultures always congealed when placed in the ice chest. All observers are in accord as to non-liquefaction of gelatin.

Difco "Bacto-tryptone." No indol was detected in twenty-four- and ninety-six-hour cultures in 1 per cent solutions of "Bacto-tryptone" in distilled water by the Steensma vanillin-HCl test as modified by Fellers and Clough (1925), and by the Ehrlich-Böhme and Goré tests as recommended by Conn et al. (1932).

Fermentation reactions. Fermentation tests were made in commercial meat extract broth and in meat infusion broth from which the muscle sugars had been removed by *Bacillus Welchii*, as describe by Randall and Hall (1921). They were tubed under vas-par[1] and sterilized in the autoclave. Ten per cent sugar solutions were prepared separately in distilled water and also sterilized in the autoclave. Just before inoculation the boiling hot sugar solutions were added to the hot sugar-free media in quantity sufficient to give a concentration of 1 per cent. Inoculations were made by pipettes from deep brain cultures that were forty-eight hours old.

Glucose, galactose, levulose, maltose, lactose, sucrose, amygdalin, salicin, and dextrin were fermented with production of acid and gas; trehalose, raffinose, xylose, rhamnose, arabinose, starch, inulin, mannitol, dulcitol, sorbitol, glycerol, and inositol were not fermented. These results agree with, and extend, those of Zeissler (1923), (1928), (1930), and Zeissler and Raszfeld (1929).

Pathogenicity

Even though all of the available strains of *"Bacillus VI"* have been under artificial cultivation for more than thirty years, we were still able to confirm von Hibler's and Zeissler's observations of pathogenicity for guinea pigs, rats, and rabbits.

Guinea pigs. Subcutaneous inoculations of 0.025 to 0.1 cc. of glucose broth cultures incubated for twenty to forty-eight hours, into guinea pigs weighing from 250 to 360 grams, caused them to die in from twenty to thirty-five hours. Doses of 0.01 cc. had no effect. Forty-eight-hour cultures were often avirulent even in larger doses.

Subcutaneous edema was the most characteristic symptom. In non-fatal cases the edema gradually subsided without necrosis.

[1] Vas-par is a mixture of equal parts vaseline and paraffine.

A few animals were seen with dyspneic convulsions and muscular spasms just prior to death.

On autopsy there was always marked subcutaneous edema and often congestion, but no emphysema or lysis. Smears from the edema fluid showed many Gram-variable, more or less granular rods with rounded ends, single, paired or rarely in chains. Spores were not seen in animals that were autopsied promptly. The viscera showed no macroscopic changes. The heart's blood was usually clotted and contained *"Bacillus VI,"* as was shown microscopically and culturally.

One guinea pig, weighing 300 grams, injected *intraperitoneally* with 1 cc. of a forty-eight-hour glucose broth culture, died in less than twenty-four hours; edema of the peritoneal cavity was marked, and the small intestines were congested.

Another guinea pig weighing 300 grams was *fed* 5 cc. of a forty-eight-hour peptone veal-infusion broth culture by pipette, without effect. A control animal of the same size injected subcutaneously with 1 cc. of the same culture died in twelve hours.

Virulent cultures were rendered avirulent by washing the organisms in three changes of 0.85 per cent NaCl solution. Heating virulent glucose-broth cultures at 80°C. for five minutes also destroyed their pathogenicity.

White rats. Animals weighing from 215 to 250 grams, inoculated subcutaneously with 0.5 cc. of glucose-broth cultures incubated for twenty-four to forty-eight hours died within twenty-four hours, and showed lesions on autopsy similar to those described for guinea pigs.

Rabbits. Rabbit 819, weighing 2300 grams, was given intravenously, 2 cc. of a forty-eight-hour glucose-broth culture of strain 2994 twice weekly for ten weeks without effect, the weight increasing during this period to 2750 grams. The blood showed agglutinin titers of 1:2000 at the end of the sixth week, and 1:2500 at the end of the tenth week.

Subsequent finding of the contaminant in this strain necessitated the immunization of another rabbit with the purified culture, but when this was attempted serious difficulty was encountered.

Ten rabbits were killed by injections of living cultures of *"Bacillus VI"* during our efforts to immunize them.

One rabbit, weighing 1350 grams, inoculated intravenously with 2 cc. of a forty-eight-hour glucose-broth culture of the purified strain, showed no symptoms up to the sixth day, when it was found dead. Another, weighing 1300 grams, given a similar dose, was apparently normal the following morning, but was seen dying at noon with a tetanus-like respiratory spasm. A third, weighing 1375 grams, inoculated intravenously with 0.01 cc. of a forty-eight-hour glucose-broth culture, was found dead on the second day. In each instance, on autopsy, the pleural cavities were filled with red fluid. There was a slight increase in fluid in the peritoneal cavities but no other macroscopic changes were noticed. Pure cultures of *"Bacillus VI"* were recovered from the hearts' blood and from the pleural fluids.

We then decided to attempt subcutaneous inoculations in which seven more rabbits were sacrificed, teaching us, first, that repeated subcutaneous inoculations of 0.01 and 0.02 cc. doses of forty-eight-hour glucose-broth cultures were apparently harmless for rabbits, second, that they were apparently without immunizing effect, and third, that succeeding doses of 0.04 cc. always killed them in two or three days, while larger doses killed in shorter periods. The only symptom noted in the rabbits inoculated subcutaneously was a localized edema but none of these animals was seen just prior to death. On autopsy, we found marked subcutaneous edema and congestion, but no macroscopic visceral changes.

Soluble exotoxin. Lehmann and Neumann (1927) stated that there were no reports on toxin production of *B. lactiparcus*, but Zeissler (1928), (1930) ranked *"Bacillus VI"* with *B. tetani* and *B. botulinus* as a toxin former.

In spite of the high pathogenicity of some of the forty-eight-hour glucose-broth cultures, several attempts to demonstrate a soluble toxin in Mandler filtrates from them resulted negatively, except for a moderate and transient subcutaneous oedema in guinea pigs inoculated with doses of from 2 to 5 cc. Negative results were also obtained with filtrates from older (eight- and ten-day) cultures.

After this, we discovered that, although young cultures in glucose broth were highly virulent, they progressively attenuated after forty-eight hours. This led us to compare the changes in acidity in peptone broth, with and without glucose, in relation to virulence.

About 200 cc. of glucose meat-infusion peptone broth, pH 6.8, under vas-par seal in a 250-cc. Erlenmeyer flask were inoculated from a deep brain culture of *"Bacillus VI."* A similar flask of meat-infusion peptone broth, pH 7.0, without added glucose, was also inoculated. Both were incubated at 37°C., and tested at intervals for changes in acidity and for virulence by subcutaneous inoculation of guinea pigs with 1 cc., as indicated in table 1.

TABLE 1

The effect of fermentation on virulence of "Bacillus VI"

INCUBATION	GLUCOSE PEPTONE BROTH—pH 6.8			PEPTONE BROTH—pH 7.0		
	pH	Guinea pig weight	Result	pH	Guinea pig weight	Result
days		*grams*			*grams*	
1	6.2	450	Found dead, 16 hours	6.8	500	Found dead, 16 hours
2	5.1	350	Found dead, 16 hours	6.8	475	Found dead, 16 hours
3	4.8	500	Edema; recovered	6.8	450	Found dead, 16 hours
5	4.6	400	No symptoms	6.8	400	Died, 24 hours

Table 1 shows that the acidity produced by fermentation of glucose was correlated with loss in virulence after the second day. This encouraged us to attempt the production of toxin in mediums to which no glucose had been added.

One guinea pig weighing 300 grams, and inoculated subcutaneously with 6 cc. of sterile filtrate from a forty-eight-hour culture in beef heart infusion peptone broth, showed marked edema at the site of inoculation on the following day. Within ten days about 50 sq. cm. of skin sloughed off; granulation soon followed, but owing to secondary infection healing was still incomplete after two months, although the animal appeared to be otherwise healthy and gained 250 grams in weight. Another guinea pig, weighing 250 grams, was similarly inoculated with 4 cc. of a filtrate from a twenty-four-hour culture, with a similar result, while

control animals inoculated with 1 cc. of the unfiltered culture died overnight.

Filtrates from twenty-four-hour veal-infusion peptone broth cultures gave still better results, killing guinea pigs weighing from 225 to 300 grams upon subcutaneous inoculation with doses of 5 cc. in about thirty-eight hours with marked subcutaneous edema. No bacteria could be seen either in smears from the lesions or in cultures from the heart's blood of the animals. Five-cubic centimeter doses of filtrates from forty-eight- and ninety-six-hour cultures produced marked edema followed by extensive sloughing with secondary infection, as is shown in figure 5, plate 1. Control tests showed that uninoculated veal infusion peptone broth was harmless in doses of 5 cc.

A rabbit, weighing 1900 grams, was found dead twelve hours after the intravenous injection of 5 cc. of the filtrate from the four-day culture. Each pleural cavity contained about 4 cc. of bloody fluid. The lungs were reddened and edematous, but there were no other significant changes.

One cubic centimeter of the same filtrate was injected intravenously into another rabbit weighing 1350 grams, without any effect. Eight days after the first injection this animal was given 5 cc. of the same filtrate intravenously; it died in about sixteen hours.

These results demonstrated the production of a weak soluble exotoxin by *"Bacillus VI,"* but not one at all comparable to those produced by *Bacillus tetani* and *Bacillus botulinus*, as intimated by Zeissler. The toxin is apparently formed during the early stages of growth in sugar-free mediums and rapidly loses strength as the cultures become older.

Immunity and serology

Zeissler (1928) (1930) stated that *"Bacillus VI"* showed strong specific agglutination; we were able after the loss of many animals during immunization to confirm this observation. We have already described the fortuitously successful production of agglutinins in rabbit 819 by intravenous inoculations of forty-eight-hour glucose broth cultures of strain 2994 before it was discovered

to be contaminated. The serum from this rabbit agglutinated antigenic suspensions of the homologous impure culture, prepared by filtering forty-eight-hour glucose broth cultures through loose cotton, in all dilutions of serum up to 1:1280; it failed to agglutinate similar preparations of *Bacillus tertius*, *Bacillus Novyi*, *Bacillus Chauvoei*, *Bacillus sporogenes*, *Bacillus botulinus*, or *Bacillus Sordellii*, in dilutions of 1 to 20 or higher. This serum failed, however, when mixed in a dose of 1 cc. with a similar amount of a forty-eight-hour glucose broth culture of strain 2994, to protect a guinea pig against fatal infection.

After many discouraging and wasteful attempts to produce a satisfactory agglutinating serum with the purified strain of "*Bacillus VI*," in which we successively tried in vain intravenous inoculations of, first large, and later small, doses of forty-eight-hour glucose broth cultures, subcutaneous inoculations of large and small doses, and subcutaneous inoculations of filtrates followed by whole cultures, we undertook to immunize a rabbit by subcutaneous injections of "anaculture" prepared according to the directions of Scott (1932) for making blackleg bacterin.

Excellent growth of "*Bacillus VI*" occurred in Scott's beef liver-muscle medium. One-hundred cubic centimeters of the fluid portion of this culture, incubated at 37°C. for seven days, were treated with 1 cc. of commercial formalin for fifteen hours at 37°C. A guinea pig weighing 325 grams was injected with 2 cc. of this preparation, without effect, after which a rabbit weighing 2075 grams was injected subcutaneously 11 times with doses doubling each time from 0.5 up to 4 cc. at intervals of a few days until a total of 32 cc. was administered without symptoms of any kind. Indeed, this rabbit nearly doubled in weight, but repeated tests of its blood serum during and after this course of injections failed to reveal agglutinins.

At this stage we learned that glucose broth cultures became avirulent after a few days; the immunization of the above rabbit was, therefore, cautiously continued, beginning with 0.25 cc. of a seven-day culture and ending in thirty-five days with 2 cc. of a three-day culture, as is shown in table 2.

Table 2 shows that the serum from the rabbit at this time agglu-

tinated the homologous culture of "*Bacillus VI*," strain 2994, in a dilution of 1:640. It likewise agglutinated each of the other strains of "*Bacillus VI*" to the same titer; it failed to agglutinate

TABLE 2

Failure of "Bacillus VI" strain 2994 anaculture to produce agglutinins, followed by successful immunization with old glucose broth cultures

DATE	RABBIT'S WEIGHT	MATERIAL INJECTED	AMOUNT	AMOUNT BLED	AGGLUTINATION TITER
1933	*grams*		*cc.*	*cc.*	
April 19	2,075			5†	Negative
April 19		Anaculture	0.5		
April 23	2,125	Anaculture	0.5		
April 27	2,150	Anaculture	1.0		
May 1	2,250	Anaculture	2.0		
May 5	2,250	Anaculture	4.0		
May 9	2,450	Anaculture	4.0		
May 13	2,450			5†	Negative
		Anaculture	4.0		
May 18	2,550	Anaculture	4.0		
June 3	3,100			10†	Negative
June 4		Anaculture	4.0		
June 11	3,300	Anaculture	4.0		
June 17	3,400	Anaculture	4.0		
June 25	3,650			8†	Negative
June 26	3,750	Culture, 7*	0.25		
July 1	3,650	Culture, 5*			
July 5	3,750	Culture, 4*	0.5		
July 9	3,650	Culture, 4*	0.5		
July 13	3,600	Culture, 4*	0.5		
July 17	3,650	Culture, 4*	1.0		
July 21	3,700	Culture, 4*	1.0		
July 26	3,775	Culture, 4*	2.0		
July 30	3,800	Culture, 4*	2.0		
August 4	3,900	Culture, 3*	2.0		
August 10	3,900			10‡	1:640

* Glucose broth culture, age in days.
† Blood obtained from ear vein.
‡ Blood obtained from heart.

the contaminant found in strain 2994, and *Bacillus fallax*, in any dilution. Similar results were secured with the serums of rabbits inoculated in a similar manner with each of the other strains.

Eight months later, fresh serum from the first rabbit failed to agglutinate *"Bacillus VI"* at 1:20. The animal was then reinjected subcutaneously with glucose broth cultures ranging progressively younger from seven to two days in 12 doses of from 1 to 2 cc. until 19 cc. had been given over a period of sixty-four days. Serum obtained six days after the last injection agglutinated the four strains of *"Bacillus VI"* equally well in all dilutions up to 1:1280. It did not agglutinate the contaminant found in strain 2994, *B. fallax*, *B. tertius*, *B. histolyticus*, *B. Sordellii*, *B. sporogenes*, *B. septicus*, *B. tetani*, nor *B. Novyi* at 1:20 or higher.

Another rabbit was successfully immunized against strain 2994 *solely* by subcutaneous injections of a similar series of aged glucose broth cultures, with the production of an agglutinating serum which gave similar results.

When the serums prepared against strains 6519, 6593, and 6594 were stored without antiseptics in the ice box for eight months, agglutinins could no longer be demonstrated at 1:20. It was still possible, however, to demonstrate protective antibodies in all of them. One cubic centimeter of each serum was mixed with fatal doses (0.05 cc.) of twenty-four-hour glucose broth cultures of each of the four strains of *"Bacillus VI."* Guinea pigs weighing 200 to 300 grams injected with such mixtures showed no effects, in contrast with control animals injected with diluted cultures without serum; three of the latter group died within twenty-four hours, and one in forty-seven hours.

Serums prepared against the Corynebacterium contaminant and *B. fallax* failed to protect similar animals against the cultures of *"Bacillus VI."*

The above experiments showed the four strains of *"Bacillus VI"* to be serologically identical and distinct from *C. lymphophilum* and *Bacillus fallax*. We have already mentioned that *Bacillus fallax* was regarded by Weinberg and Séguin (1917) as probably identical with von Hibler's *"Bacillus VI,"* but one of us (D.) will show in a separate paper that *B. fallax* is not only serologically, but also morphologically, culturally, and pathogenically a distinct species.

THE IDENTITY OF "BACILLUS VI" WITH KLEIN'S BACILLUS CARNIS

One of our objectives was to establish a valid specific name for "*Bacillus VI*," and, since several names had already been proposed, we were apparently confronted with the necessity of choosing one which deserved consideration from the viewpoint of priority. But as it turned out, an organism apparently identical with "*Bacillus VI*" had already been described under the name *Bacillus carnis* by Klein (1903, 1904) several years before von Hibler first used the designation "*Bacillus VI*," and, of course, long before any of the names more recently suggested, such as "*Hiblerillus sextus*" (Heller) and *Bacillus lactiparcus* (Lehmann and Süssmann). We were, furthermore, able to trace the origin of our last strain of "*Bacillus VI*" back to Klein himself, and this, in our opinion, lends further support to the propriety of using Klein's name.

It will be recalled that Lehmann and Neumann (1927) recorded Zeissler's isolation and identification of "*Bacillus lactiparcus*" from an impure culture of Klein's "*Bacillus enteritidis sporogenes*" which had been sent by Klein to von Hibler in 1898, and described by von Hibler (1908) as "*Art IV*." All of Zeissler's papers published alone and with Raszfeld since 1928 refer to this strain. Upon receiving it from Dr. Zeissler and learning its history, we decided to review carefully Klein's voluminous published reports upon various bacteriological problems during the decade and a half in which he was particularly active. In so doing, we came across Klein's (1903, 1904) descriptions of *Bacillus carnis*, which he had isolated from contaminated beef infusion. It is not necessary to repeat here the characteristics which Klein noted. Suffice it to say that they check closely with those of "*Bacillus VI*," with two exceptions.

The first lies in Klein's description of *Bacillus carnis* as an obligate anaerobe; but it is significant that von Hibler overlooked the micro-aerophilic growth of "*Bacillus VI*," observed by Zeissler and ourselves. The second consists in his failure to demonstrate pathogenicity in guinea pigs by intraperitoneal injections,

which may be explained by uncontrolled variations in virulence such as we encountered in our own virulence tests.

We were thus led to accept *Bacillus carnis* (Klein) as the valid scientific name for von Hibler's "*Bacillus VI.*"

DIFFERENTIAL PROPERTIES OF THREE SPECIES OF MICRO-AEROPHILIC BACILLI

Bacillus carnis is the third micro-aerophilic bacillus which we are now able to distinguish, the other two being *Bacillus histolyticus* (Weinberg and Séguin) and *Bacillus tertius* (Henry). Although all of these were originally described as obligate anaerobes, they all show delicate aerobic growth upon the surface of solid mediums. None of them produces spores aerobically; all produce them anaerobically. In *B. histolyticus* the spores are oval and subterminal; in *B. tertius* they are elongate and terminal; in *B. carnis* they are oval to elongate and mostly subterminal; *B. carnis* and *B. histolyticus* are hemolytic and pathogenic; *B. tertius* is non-hemolytic and non-pathogenic. *B. tertius* and *B. carnis* are actively fermentative, non-putrefactive and non-gelatinolytic; *B. histolyticus* is non-fermentative, putrefactive and gelatinolytic.

These differences may be expressed in the form of an artificial key as follows:

Micro-aerophilic, growing delicately as facultative aerobes, but sporulating only under anaerobic conditions.
  Spores subterminal.
    Hemolytic.
      Iron brain blackened; tyrosine produced.
        Gelatin liquefied.
          Glucose, lactose, and mannitol not fermented ..... *B. histolyticus*[1]
      Iron brain not blackened; no tyrosine produced.
        Gelatin not liquefied.
          Glucose and lactose fermented; mannitol not fermented ..... *B. carnis*[1]
  Spores terminal, elongate.
    Non-hemolytic.
      Iron brain not blackened; no tyrosine produced.
        Gelatin not liquefied.
          Glucose, lactose, and mannitol fermented ..... *B. tertius*

[1] Pathogenic.

SUMMARY

1. Four separate strains of von Hibler's *"Bacillus VI"* were studied with reference to morphologic, cultural, pathogenic, and serologic properties.

2. Three of these strains were received as pure cultures; one was contaminated by an organism resembling *Corynebacterium lymphophilum* (Torrey *emend.* Bergey, et al.).

3. All of the pure strains of von Hibler's *"Bacillus VI"* were shown to be identical in all of their properties and to constitute a distinct species of micro-aerophilic bacilli.

4. Von Hibler's *"Bacillus VI"* is identical with *Bacillus carnis (Klein)*, and the latter name should be used in preference to *"Hiblerillus sextus (Heller)"* and *"Bacillus lactiparcus (Lehmann and Süssmann)"* on the ground of priority.

5. The following observations of Zeissler relating to von Hibler's *"Bacillus VI"* are hereby confirmed and restated in terms of *Bacillus carnis.*

a. Bacillus carnis was a pathogenic non-proteolytic component of a culture of *"Bacillus enteritidis sporogenes"* sent by Klein to von Hibler and described by the latter as *"Bacillus IV."*

b. Bacillus carnis is a micro-aerophilic, not an obligate, anaerobe.

c. Bacillus carnis produces a weak filterable exotoxin.

d. Bacillus carnis produces highly specific agglutinins in rabbits.

6. *Bacillus carnis* is hemolytic, both aerobically and anaerobically, and in plain and in glucose blood agar.

7. Agglutinins are produced with difficulty using young toxic cultures, but easily using old non-toxic cultures.

8. Specific antiserums were produced which neutralized all of the cultures of *B. carnis*, but not those of other pathogenic anaerobic bacilli.

We are indebted to Miss Elizabeth O'Toole for the flagellar stain, and to Mr. Glen Mills for the photographs.

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PLATE 1

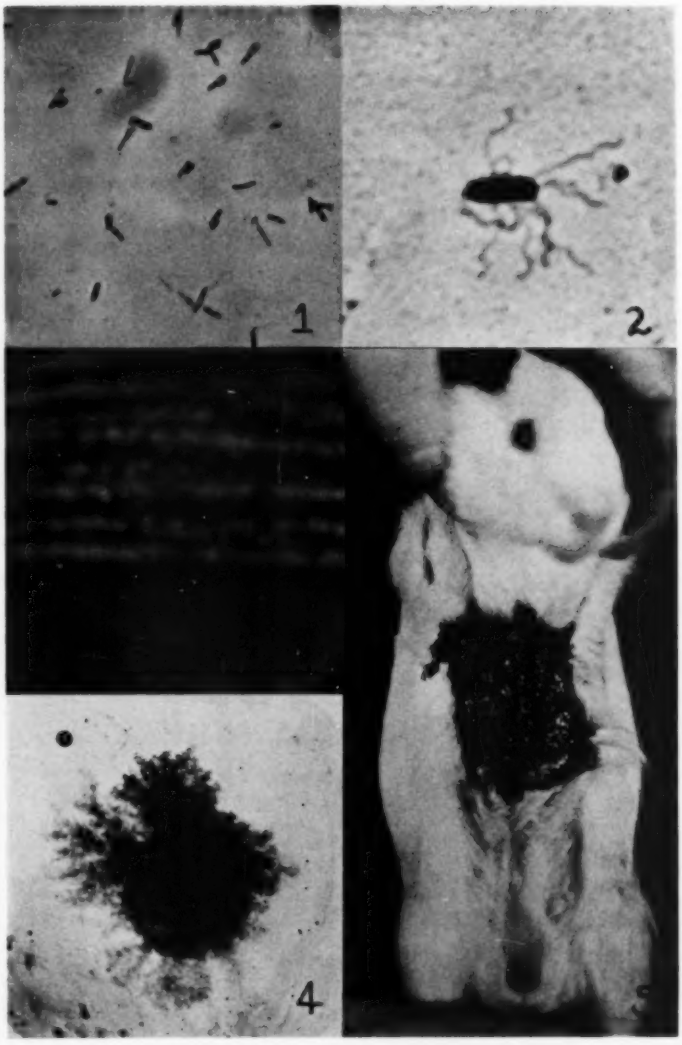
FIG. 1. Vegetative rods and spores of *Bacillus carnis*, strain 2994, stained by Gram's method from a forty-eight-hour anaerobic blood agar slant. × 1000.

FIG. 2. *Bacillus carnis*, strain 6594, from twenty-two-hour anaerobic blood agar slant with flagella stained by Bailey's method. × 3300.

FIG. 3. Section of streaked aerobic blood agar plate showing hemolysis due to forty-eight-hour growth of *Bacillus carnis*, strain 2994. As in the original plate, the colonies are almost invisible.

FIG. 4. Colony from thirty-seven-day culture of Bacillus carnis, strain 2994 in deep 1 per cent agar. × 10.

FIG. 5. Three-hundred-gram guinea pig, ten days after the subcutaneous injection of a Mandler filtrate of a four-day culture of *Bacillus carnis*, strain 2994, in veal infusion peptone broth under vas-par seal.



(Ivan C. Hall and Nicholas D. Duffett: Identification of von Hibler's "Bacillus VI")

A SPECIES OF SALMONELLA PRODUCING A WATER-SOLUBLE PIGMENT

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In the course of an investigation of natural paratyphoid infection in various rat colonies an organism was isolated which produced a brilliant, yellow, water-soluble pigment. Aside from this characteristic, it was indistinguishable from strains of *S. enteritidis* which were isolated from endemics in other colonies of rats. The organism to which we refer appeared as a typical smooth colony surrounded by a yellow zone when streaked on agar plates. At first, it was suspected of being *Pseudomonas fluorescens*. This however was not so, as the organism was subsequently found to possess the characteristics of *S. enteriditis*.

PROPERTIES OF S. ENTERITIDIS-YELLOW

1. *Colonial appearance.* Smooth, indistinguishable from typical *S. enteritidis*.

2. *Microscopic appearance.* Actively motile, Gram-negative rod.

3. *Biochemical reactions* (table 1).

TABLE 1

	GLUCOSE		LACTOSE		SALICIN		SUCROSE		ARABINOSE		XYLOSE		INOSITE	
	A	G	A	G	A	G	A	G	A	G	A	G	A	G
S. enteritidis	+	+	−	−	+	+	−	−	+	+	+	+	−	−
S. enteritidis-yellow	+	+	−	−	+	+	−	−	+	+	+	+	−	−

This organism, like *S. enteritidis* liberated H_2S causing the blackening of lead acetate, produced no change in litmus milk, did

not liquefy gelatin or Loeffler's coagulated serum, caused green hemolysis on blood plates and grew very slowly anaerobically.

4. *Reciprocal agglutination.* Table 2 shows that the pigmented organism is serologically very closely related to *S. enteritidis.*

TABLE 2

ANTIGEN	ANTISERUM	TITRE	ANTIGEN	ANTISERUM	TITRE
Enteritidis	Enteritidis	130,272	Yellow	Aertrycke	64
Aertrycke	Aertrycke	1,024	Aertrycke	Enteritidis	16
Yellow	Yellow	4,096	Enteritidis	Aertrycke	256
Enteritidis	Yellow	130,272	Aertrycke	Yellow	8
Yellow	Enteritidis	4,096			

PATHOGENICITY

The pathogenicity of this organism was tested in rats. The following doses were administered to animals weighing approximately 100 grams.

One rat received 1.0 cc. of a twenty-four hour broth culture intraperitoneally.

One rat received 0.5 cc. of a twenty-four hour broth culture intraperitoneally.

One rat received 0.1 cc. of a twenty-four hour broth culture intraperitoneally.

Three rats received 0.3 cc. each of a twenty-four hour broth culture *per os.*

The rats which received 1.0 cc. and 0.5 cc. intraperitoneally died within eighteen hours after infection. The rat receiving 0.1 cc. died after forty-eight hours. Typical smooth organisms having all the properties of the original culture, including chromogenicity, were recovered from the liver, spleen and feces in every case. Two of the rats which received the bacteria *per os* did not die, and were autopsied after two weeks. Here again, the same organisms were recovered from their livers, spleens and feces. The third, which died at the end of two weeks was not autopsied because of advanced post mortem decomposition. The pathogenicity of this organism compares favorably with that of other strains of *S. enteritidis* isolated from other colonies of rats.

THE PIGMENT

The pigment is produced most abundantly in the presence of a certain peptone (Fairchild Brothers and Foster) in a medium containing 1 per cent peptone, 0.5 per cent NaCl and 2 per cent agar. The meat extract or infusion used seems to have no influence on pigment production. The maximum quantity of pigment is produced by incubating cultures on agar plates at 37°C. for twenty-four hours. If the plates remain in the incubator after this period the pigment tends to fade and at the end of forty-eight hours is scarcely detectable. In fluid media the pigment production proceeds slowly reaching a maximum after about three days, but never approaching the quantity obtained on solid media.

The pigment is water-soluble and diffuses into the surrounding agar with an appearance similar to that of a *Ps. fluorescens* culture. It differs, however from the pigment of *Ps. fluorescens* in that it is a deeper yellow and does not fluoresce. Spectroscopically, it does not exhibit any characteristic lines but merely a complete cut-off in the blue-violet region. It is easily extracted by triturating the agar upon which the organism has grown and allowing the pigment to diffuse out into distilled water. The pigment is dialysable, not volatile, resists boiling, and strong acids and alkalies. It is insoluble in benzene, chloroform, or ether. In acid the color disappears but is restored by the addition of alkali.

VARIANTS

A few of the dissociants of this organism were studied in order to determine whether colonial variation induces any change in pigment production. The following variants were studied: S, the original smooth form; F, flat or dry form; R, rough type; O, mucoid type. All these colony types produced the pigment.

THE PIGMENT PRECURSOR

The substrate which is necessary for the production of the pigment seems to occur infrequently. Substances were tested for the presence of the substrate in the following manner. A sample

was added to a blank medium consisting of neo-peptone (1 per cent), sodium chloride (0.5 per cent) and agar (2 per cent). This medium by itself was incapable of eliciting the chromogenic properties of the organism. A large number of peptones and other substances were tested in this way. Peptone prepared by digesting beef with trypsin (Fairchild) and also one sold by Eimer and Amend caused marked color production. Most of the other substances tested did not possess this property and the few that did, produced a minute quantity of pigment. The latter were proteose-peptone (Difco), Parke Davis peptone and Merck's pepsin and pancreatin. In an attempt to determine the nature of the precursor one sample of Fairchild's peptone was ashed, and another hydrolyzed in 5N HCl for forty-eight hours so that no biuret material was left. The ash did not contain the precursor, but the hydrolysate did. This indicates that it is neither a mineral nor a protein breakdown product but probably a simple organic compound. Amino acids, (cystine, tyrosine, tryptophane, methionine, argenine and histidine) were added in excess to the blank medium but no color was produced. The peptone was refluxed with each of the following solvents: acetone, absolute ethyl alcohol, benzene, chloroform, ether, methyl alcohol. Only in the ethyl and methyl alcohol extracts was any of the precursor found. Attempts were made to precipitate the protein-like substances with trichloracetic acid, lead acetate, phosphotungstic acid, and alcohol. In all instances complete precipitation of the protein material was associated with a loss of the precursor. Norite, if used in sufficient quantity removed all of the precursor but kieselguhr, Fuller's earth, kaolin, and bentonite did not adsorb the desired substance in appreciable amounts. At the present time the only way of demonstrating the chromogenic power of the organism is by the use of the proper peptone.

OCCURRENCE OF CHROMOGENS AMONG THE SALMONELLAS

It was of interest to determine if various other cultures of the Salmonella group possess the same pigment when grown on the same medium. Accordingly, the following strains were studied.

S. aertrycke, European suipestifer, American suipestifer (obtained through the courtesy of Dr. E. O. Jordan), *S. aertrycke*, *S. enteritidis*, Hog cholera, *S. paratyphi*, *S. Schottmuelleri*, and the Newport, Derby, Voldagsen, Reading, and Stanley types (obtained through the courtesy of Dr. K. Landsteiner), and *S. enteritidis* and *S. aertrycke* (obtained through the courtesy of Dr. L. T. Webster). The results with the above cultures were identical in that none produced the yellow pigment. Similar findings were obtained with 50 variants of *S. aertrycke* which had been isolated in this laboratory. It would appear therefore, that the production of soluble pigment by the genus Salmonella is a rare phenomenon. In addition a few laboratory cultures of *Eb. typhi*, *S. paratyphi* and *S. Schottmuelleri* were tested with negative results.

SUMMARY AND DISCUSSION

An organism producing a soluble pigment on certain media was isolated from the feces of a rat with enteric infection. Since this chromogen aside from its color, is indistinguishable from typical cultures of *S. enteritidis* constantly isolated from rats, it is reasonable to suppose that it is a variant of the others. Although it is impossible to state with absolute certainty that any given bacterial modification is a mutation, the chromogenic properties of this culture may possibly represent one. In the first place the change is rare. The organism was found in the smooth or naturally occurring pathogenic form. The property of pigment production is fixed; all the variants thus far studied have it and it is not lost in passage through animals. The organism gives rise to the same types of variants as do all smooth Salmonellas which we have studied.

Ordinary dissociation is probably not a mutation since many dissociants have been shown to be capable of reversion. Then again, the variants through the Salmonella group are all very much alike, i.e., they are composed of analogous smooth, rough, mucoid and flat forms. But the production of a pigment which is fixed for all the variants seems to be a superimposed property which is distinct from variation and is analogous to the

development of a new fermentation reaction which is common to all the variants and marks the origin of a new species.

Since *S. enteritidis* is a frequent natural pathogen in rats, one cannot always be sure that an organism isolated from a rat or mouse has arisen from an injected culture or whether it is a contaminant. As the chromogen is a typical *S. enteritidis* and in addition is easily differentiated from the common form it may be of use in studies on the variation of the Salmonella group *in vivo*, as well as in those on the relation of nutrition to infection, where the identity of an injected strain must be established beyond doubt.

We are greatly indebted to Dr. Slanetz of the Department of Animal Care and Professor Zucker of the Department of Pathology for kindly advice and the use of materials.

THE PERSISTENCE OF AVIAN TUBERCLE BACILLI IN SOIL AND IN ASSOCIATION WITH SOIL MICRO-ORGANISMS[1, 2]

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The purpose of these investigations was to obtain information on the death rate of tubercle bacilli in soil. Undoubtedly many tubercle bacilli become lodged in soil by animal contamination. It is possible, therefore, that tubercle bacilli surviving for a long period in soil may play a rôle in the spread of disease. The amount of infection possible would be proportional to the time of survival. Schalk (1928) has presented evidence of soil transmission of avian tubercle bacilli.

METHOD OF ISOLATING TUBERCLE BACILLI FROM HIGHLY CONTAMINATED MATERIALS

The method of study used to obtain the information in this paper has involved infecting the soil with tubercle bacilli and obtaining a plate count of the tubercle bacilli periodically. A plating method satisfactory for isolating Mycobacteria from many contaminated materials was developed. Tubercle bacilli can be isolated from among Gram-negative bacteria by treatment with alkali, acid or other chemical agents to which Gram-negative bacteria are sensitive. On the other hand, they can be separated from Gram-positive bacteria by culturing on crystal violet medium. Crystal violet retards Gram-positive bacteria. When a mixed culture containing Mycobacteria is treated with sodium hydroxide and plated on glycerol agar containing crystal violet,

[1] Journal Series Paper, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

[2] These investigations were supported by a grant from the National Research Council and carried out under the auspices of Committee on the Microbiology of the Soil.

the Mycobacteria may thus develop in colonies and the plates remain relatively free from other forms. The combination of alkali treatment and culturing on crystal violet medium has been found exceedingly effective in retarding development of most common soil-microörganisms other than certain soil acid-fast bacteria. All acid-fast bacteria cannot be isolated by this method. Several strains worked with were too sensitive to alkali. Pathogenic and non-pathogenic strains may or may not be isolated by this procedure, the success depending upon the physiology of the organism dealt with. Certain soil Mycobacteria have been easily isolated by the method. *Avian 531* was easily isolated but the *H37* human strain and the *Ravenel* bovine strain of tubercle bacilli were found more difficult to deal with because they were not so markedly alkali-resistant.

An important factor in the success of this plating method has probably been the fact that the inoculum was always put on the surface of an agar plate in 1 cc. of a very dilute agar. The bacteria were thus always at the surface of the agar imbedded in a shallow layer of nutrient medium. Satisfactory plates were obtained only after the use of a dilute agar blank was adopted.

The medium used was varied somewhat during the course of the experiments. Slight changes in composition were not observed to give very different results. The addition of yeast, egg yolk, and potato extracts was found to promote growth. One batch of medium was used for each experiment to avoid danger of variation.

Medium used for plating

Glycerol	100	cc.
Peptone	5	grams
Beef extract	3	grams
Yeast extract	2	grams
Extract of two egg yolks		
Extract of 200 grams potato		
K_2HPO_4	0.6	gram
KH_2PO_4	0.2	gram
Agar	40	grams
0.6 cc. of 1 per cent alcoholic crystal violet		
Water to make 2 liters of medium		

Dilution blanks

One part of above medium
Four parts of water

The egg and potato extracts were prepared by boiling the egg yolk and sliced potato in a liter of water for fifteen minutes and filtering off the solids. The agar medium and dilution liquid were sterilized in the autoclave. The melted agar was cooled to 45°C. and poured into sterile Petri dishes. After the agar solidified, the Petri dishes were placed in an autoclave and the temperature maintained at about 70°C. for an hour. The autoclave was not opened until the plates had cooled to room temperature. This partial sterilization of the poured plates eliminated the majority of contaminating organisms which were sucked into the plates while the agar was cooling. The partial sterilization of poured plates probably would be unnecessary in a laboratory protected from dust. The dilution blanks were heated to liquefy and cooled to 40°C. The thoroughly shaken material containing the tubercle bacilli was alkali-treated, neutralized, and diluted as for the ordinary plate count, the dilution agar being used as a sterile dilution medium. One cubic centimeter of a desired dilution was placed on the surface of a prepared agar plate and the dish quickly rotated to spread the inoculum over the surface. The plates were inverted in about half an hour, placed under bell jars and incubated at 37°C. The colonies were counted after three weeks of incubation.

EFFECT OF PURE CULTURES OF SOIL MICROÖRGANISMS ON AVIAN TUBERCLE BACILLUS 531 IN SOIL

Fifty-gram samples of soil were placed in 250 cc. flasks and sterilized in the autoclave for one hour; also, soil containing 20 per cent of horse manure was similarly sterilized. Calcium carbonate was added to all soil. All flasks were equally inoculated with *Avian 531*. The following outline indicates the additional inoculations made into some of the flasks: (1) Pure culture, *Avian 531*, tubercle bacillus; (2) *Avian 531* and *B. megatherium*:

(3) *Avian 531* and *A. aerogenes*; (4) *Avian 531* and soil anaerobe; (5) *Avian 531* and the fungus, *Verticillium chlamydosporum*.

The water content of the soil was adjusted to about 25 per cent and stoppers were inserted above the cotton plugs to prevent evaporation. The flasks were kept at room temperature which ranged about 25°C. The soil was treated for five minutes with 2 per cent sodium hydroxide before plating.

TABLE 1

Plate count of Avian 531 in soil associated with soil microörganisms

DAYS OF INCUBATION	PURE	WITH B. MEGATHERIUM	WITH A. AEROGENES	WITH ANAEROBE	WITH FUNGUS
0	68,000	68,000	68,000	68,000	68,000
17	181,000	525,000	46,000	430,000	10,000
67	980,000	340,000	310,000	400,000	3,000
97	5,750,000	2,950,000	810,000	2,300,000	39,000

TABLE 2

Plate count of Avian 531 in manured soil associated with soil microörganisms

DAYS OF INCUBATION	PURE	WITH B. MEGATHERIUM	WITH A. AEROGENES	WITH ANAEROBE	WITH FUNGUS
0	68,000	68,000	68,000	68,000	68,000
17		450,000	475,000	225,000	12,000
67	1,250,000	660,000	750,000	35,000	3,500
97	21,000,000	11,000,000	18,000,000	50,000	3,000

The pure culture count increased so notably that it seems probable that multiplication occurred. The observed increase is about 100 fold after three months. The soil containing *A. aerogenes* and *B. megatherium*, maintained approximately the same tubercle bacillus population as the pure culture. No significant difference between the pure culture count and the count of *Avian 531* was noted associated with the anaerobe in soil, but the count of the tubercle bacilli in manured soil was low in the presence of the anaerobe. The manure probably made conditions more suitable for the anaerobe. The fungus was decidedly inhibitory to *Avian 531* under the conditions of this experiment.

The plate counts of *Avian 531* after culturing with various pure

cultures of bacteria and actinomycetes in soil for one month are given in table 3. Fifty-gram samples of soil were sterilized and inoculated with equal amounts of an *Avian 531* suspension. Calcium carbonate had been added to the soil. Some of the flasks were also inoculated with a pure culture of another microörganism as indicated in table 3. All the flasks were stoppered with cotton plugs and were kept in a very humid incubator at 28°C. The plate count of tubercle bacilli at the time of inoculation was 870,000 per gram of soil. At the end of one month all samples were plate-counted.

No plate counts were made of any organism other than the tubercle bacillus. It is quite certain that the soil microörganisms

TABLE 3

Avian 531 with soil bacteria and actinomycetes

MICROÖRGANISMS IN SOIL	PLATE COUNT OF AVIAN 531 AFTER ONE MONTH (DUPLICATE COUNTS)	
Avian 531 and *Azotobacter vinelandi*	5,000,000	6,200,000
Avian 531 and *Serratia marcesens*	11,000,000	3,200,000
Avian 531 and *Pseudomonas fluorescens*	1,200,000	600,000
Avian 531 and *Proteus vulgaris*	3,600,000	3,600,000
Avian 531 and *Bacillus subtilis*	3,800,000	2,500,000
Avian 531 and *Actinomyces Lipmanii*	1,400,000	1,900,000
Avian 531 and *Actinomyces violaceus-rubes*	2,100,000	1,600,000
Avian 531 and *Actinomyces pheochromogenus*	5,400,000	4,300,000
Avian 531 (pure culture)	8,600,000	6,300,000

developed abundant populations. Inoculations were large and were made from fresh growths on agar slants. The counts of *Avian 531* from duplicate flasks checked satisfactorily. These counts were not depressed markedly by any of the soil microorganisms. The pure culture count is slightly higher than the others, but the difference is not of a significant order.

Table 4 gives the plate counts of *Avian 531* after being cultured for one month with pure cultures of fungi and with known mixtures of fungi and bacteria. The study was conducted with soil and with soil enriched by 2 cc. of nutrient broth and 0.02 gram of sucrose per 50 grams of soil. Calcium carbonate was added to all soil to control the reaction. The sterile soil samples were also

inoculated with *Avian 531* as in the previous experiments and the additional inoculations made from pure cultures of fungi and bacteria suspended by shaking with sterile gravel and water. The infected soil was kept at 28°C. in a humid incubator. The flasks were plugged with cotton.

TABLE 4

Avian 531 with fungi and with known mixtures of fungi and bacteria

ORGANISMS INOCULATED INTO SOIL	PLATE COUNT OF AVIAN 531 AFTER ONE MONTH	
	In soil enriched by 2 cc. of nutrient broth and 0.02 gram of sucrose per 50 grams of soil	In soil
Avian 531 (pure culture)	21,000,000	600,000
Avian 531 and *Humicola*	1,200,000	2,400,000
Avian 531 and *Aspergillus niger*	9,600,000	13,000,000
Avian 531 and soil penicillium	2,200,000	3,900,000
Avian 531 and *Trichoderma 105*	340,000	2,200,000
Avian 531 and *Mucor Rouxii*	*	4,500,000
Avian 531 and *Rhizopus 120*	6,900,000	5,800,000
Avian 531 and *Aspergillus flavus*	*	4,000,000
Avian 531 and *Verticillium chlamydosporum*	900,000	3,200,000
Avian 531, *Aspergillus flavus*, *A. aerogenes* and *B. megatherium*		3,000,000
Avian 531, *Trichoderma 105*, *A. aerogenes* and *B. megatherium*		3,100,000
Avian 531, *A. aerogenes* and *B. megatherium*		5,400,000
Avian 531, all fungi listed above, *A. aerogenes*, and *B. megatherium*		400,000
Avian 531 and all fungi listed above	*	*
Avian 531, soil penicillium, *A. aerogenes*, and *B. megatherium*		1,500,000

* Fungi overgrew plates so badly that no counts were obtained.

Macroscopic examination of the flasks revealed that the enriched soil had a greater abundance of fungus mycelium. The presence of *A. aerogenes* and *B. megatherium* reduced the amount of fungus mycelium.

The soil was treated for ten minutes with 2 per cent sodium hydroxide before plating. The plate count of *Avian 531* at the time of inoculation was 10,000,000 per gram of soil.

Considerable variation occurred in the plate counts, but in general, the numbers of *Avian 531* were not markedly reduced. At least, the data showed conclusively that a large percentage of the tubercle bacilli survived, even when associated with complex mixtures of fungi and bacteria. While the data as a whole indicate conclusively the long viable period of *Avian 531*, they have less value in indicating the effect of a single organism on the death rate. The plate count does not warrant conclusions from a single determination.

AVIAN 531 IN TOLUOL STERILIZED SOIL

Extensive studies were made of the death rate of *Avian 531* in soil partially sterilized by toluol. Plate-counting of tubercle bacilli in toluol-sterilized soil was less difficult than in unsterilized soil because the fungi which overgrew many plates were partially eliminated and the soil acid-fast bacteria, difficult to distinguish from tubercle bacilli, were destroyed. Five cubic centimeter portions of toluol were added to 250 cc. flasks containing 50 grams of soil. The flasks were allowed to remain stoppered for twenty-four hours. The stoppers were then replaced by sterile cotton plugs and aeration was permitted for one week at 37°C. A heavy suspension of *Avian 531* was then prepared by shaking the bacteria from a glycerol agar slope with glass beads. All the flasks were then equally inoculated with the same amount of suspension and the water content adjusted to about 25 per cent. Rubber stoppers were used to retain the moisture. Control flasks were similarly treated except that inoculation with tubercle bacilli was omitted. The acid-fast bacteria were counted by plating at various intervals. The data are presented in table 5. At some of the intervals the total bacterial count of the soil was also determined by plating on nutrient agar.

The soil was treated with 2 per cent sodium hydroxide for thirty minutes in the *Avian 531* count procedure. No acid-fast bacteria appeared on the plates from the control soil. Undoubtedly the death rate of the tubercle bacillus was not rapid in this soil. The slight rise in count after the twenty-nine days of incubation scarcely indicated that an actual increase in numbers

occurred. Recovery of the tubercle bacilli became more and more difficult as the number of tubercle bacilli decreased and the soil bacteria increased. Although the observations made with the toluol-sterilized soil are not equivalent to what might have occurred in unsterilized soil, the results prove that tubercle bacilli are capable of existing in soil in competition with many soil microörganisms for at least two months.

TABLE 5

AVIAN 531 IN TULUOL STERILIZED SOIL		TOTAL NUMBER OF BACTERIA IN SOIL
Days after inoculation	Number of Avian 531 per gram of soil	
0	53,000	
7	160,000	8,500,000
29	272,000	
67	3,000	16,000,000

The results of table 5 are quite typical of those from several similar experiments.

TABLE 6

Survival of Avian 531 in toluol sterilized soil under various conditions

NUMBER OF DAYS AFTER INFECTION	NUMBER OF TUBERCLE BACILLI PER 1/1000 GRAM OF SOIL											
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
0	78	78	78	78	78	78	78	78	0	16	78	0
17	2	17	6	24	16	22	38	69	0		88	0
30	5	125	150	16	0	13	12	20		240	9	0
48	22	16	90	1	40	1	18	28		325	1,900	
99	5	2	0			21	0	3	0	40	*	0

* Bad contamination of plates.

EFFECT OF VARIOUS ORGANISMS AND VARIOUS CONDITIONS ON SURVIVAL OF AVIAN 531 IN TOLUOL-STERILIZED SOIL

Toluol-sterilized soil was prepared as in the previous experiment. All flasks except the controls, 9 and 12, which were not infected, and 10, which received 1 cc. of *Avian 531* suspension,

received 5 cc. of *Avian 531* suspension. Modifications are indicated in the following list which gives the various treatments.

1. Five cubic centimeters of *Avian 531*.
2. Five cubic centimeters of *Avian 531* and inoculated with *Colpoda cuculus*.
3. Five cubic centimeters of *Avian 531* and inoculated with *Colpoda Steinii*.
4. Five cubic centimeters of *Avian 531* and inoculated with mixed soil ameba culture.
5. Five cubic centimeters of *Avian 531* and incubated at 37°C.
6. Five cubic centimeters of *Avian 531* and 0.05 gram of ammonium sulfate added per 50 grams of soil.
7. Five cubic centimeters of *Avian 531*; 4 cc. less water than in first flask.
8. Five cubic centimeters of *Avian 531*; 5 cc. more water than in first flask.
9. Control, no *Avian 531* added.
10. *Avian 531* in sterile soil. Soil sterilized in autoclave. One cubic centimeter of *Avian 531* added.
11. Five cubic centimeters of *Avian 531* per 50 grams of soil. This infected soil was placed in a pot in the field.
12. Control (no *Avian 531*) placed in pot in the field.

The water content of the soil was about 25 per cent in all the flasks except 7 and 8. The soil of series 7 had about 17 per cent water and the soils of series 8 contained about 35 per cent water. All the flasks were stoppered throughout the course of the experiment to prevent loss of water. Samples 11 and 12, however, were exposed to field conditions. The weather was excessively rainy during the first few weeks of the experiment and then became hot and dry. Toluol sterilization eliminated protozoa, which were, therefore, present only in the soil to which cultures were introduced. All samples but 5, 11, and 12 were kept at room temperature.

Table 6 gives the results of the *Avian 531* plate counts. The soil samples were treated with 2 per cent sodium hydroxide for thirty minutes before plating. Counts are given in thousands per gram of soil.

The data are much too variable for detection of differences in death rate as a result of the different treatments. None of the protozoa altered the death rate sufficiently for detection. Varying moisture, temperature and nitrogen content did not alter the viability. The results with *Avian 531* in the field are most irregular but it is certain that large numbers survived six weeks. The results as a whole leave no doubt that *Avian 531* is able to survive for a considerable period under a variety of conditions. The pure culture, sample 10, had a quite significant increase in numbers.

SURVIVAL OF AVIAN 531 IN UNSTERILIZED SOIL

Although the study of the survival of *Avian 531* in unsterilized soil presents serious difficulties, some degree of success has been

TABLE 7

Survival of Avian 531 in unsterilized soil

DAYS AFTER INOCULATION	NUMBER OF AVIAN 531 PER GRAM OF SOIL
0	56,000,000
3	65,000,000
13	67,000,000
23	14,000,000
31	11,000,000
44	3,500,000
59	2,700,000

achieved. Early attempts failed because the amounts of *Avian 531* added to the soil were too small and the medium was not suitable for the best plating results with tubercle bacilli. The death rates of large inocula of tubercle bacilli have been determined by plating the infected soils on the medium described in this paper. The soil was treated with 2 per cent sodium hydroxide for thirty minutes before being plated. The count of *Avian 531* was so high in these studies that there was no danger of soil acid-fast bacteria being counted as tubercle bacilli. Control samples of soil inoculated with heat-killed tubercle bacilli yielded only about 10,000 acid-fast bacteria per gram of soil. The soil was moist. Fifty gram samples were kept in 250 cc. flasks. The

flasks were kept in a very humid incubator at 28°C. Conditions were favorable for great activity of soil microörganisms.

Another experiment has given results verifying the death rate observed in table 7, and has added a few observations on the death rate under somewhat varied conditions. Tubercle bacilli were inoculated into flasks of soil as in the previous experiment and the plate count determined periodically. The soil was treated with 2 per cent sodium hydroxide for five minutes before plating.

TABLE 8

Survival of Avian 531 in unsterilized soil

DAYS AFTER INOCULATION	A	B	C	D	E
0	6,000,000	6,000,000	6,000,000	6,000,000	6,000,000
5	8,500,000				
12	3,500,000				
19	1,200,000				
33	800,000	660,000	530,000	10,000	20,000

A. Incubated at 28°C.; water content about 25 per cent.

B. Incubated at 37°C.; water content about 25 per cent.

C. Incubated at room temperature (about 20°C.); water content about 25 per cent.

D. Samples allowed to air dry at 28°C. in an incubator with no humidifier.

E. Incubated at 28°C. in a test tube, depth of soil being about 5 inches. Water was added until the water level was above the soil surface.

Table 7 and column A of table 8 represent counts made under very similar conditions. The numbers of tubercle bacilli decreased consistently with time of culture in the soil. The rate of decline in numbers was higher than that noted generally in pure culture associations. The rate is so slow, however, that it seems justifiable to conclude that the tubercle bacilli would survive for many more months. According to the figures of table 8 the death rate is about the same at 37°C. and at room temperature (about 20°C.) as at 28°C. This organism has an optimum growth at 37° or slightly higher and will grow fairly well at 28°C. The growth rate at room temperature is extremely slow. At room temperature, survival must be due chiefly to the inoculated

cells maintaining their viability rather than to the production of new cells at a rate sufficiently high to maintain the observed population.

In soil allowed to air-dry at 28° and in soil covered with water, the death rate was much accelerated. Even these unusual treatments did not completely destroy the tubercle bacilli. Control plates showed that soil acid-fast bacteria were not confused with the tubercle bacilli at the low dilutions.

DISCUSSION

Avian 531 increased in population when inoculated into sterile soil and when grown in soil in association with some pure cultures of bacteria. In association with other pure cultures of microorganisms a slow death rate has been noted. The difference in death rates caused by different organisms was not extensively studied. The plate count of tubercle bacilli decreased consistently in soil sterilized by toluol and in unsterilized soil. This death rate is due to the presence of the complex microbiological population but no evidence points to particular organisms as chiefly responsible for the destruction of the tubercle bacilli or to the mechanism of destruction.

SUMMARY

1. A method suitable for plate-counting of *Avian 531* tubercle bacilli in the presence of complex microbiological populations was developed.

2. The *Avian 531* tubercle bacillus was found to multiply in sterile soil and when associated with pure cultures of some soil bacteria. Under the conditions of this same experiment, a fungus checked development of *Avian 531*, especially in manured soil.

3. In general, bacteria, fungi and actinomycetes did not markedly depress the number of tubercle bacilli in soil when cultured in association with the tubercle bacilli.

4. *Avian 531* tubercle bacilli survived in toluol-sterilized soil for long periods under a variety of conditions. Recovery from toluol-sterilized soil was made after three months. The death rate was slow.

5. *Avian 531* tubercle bacilli were slowly destroyed in soil which had not been sterilized. The plate count numbers were reduced to about one-sixth of the original counts in one month. If the same death rate continued, the tubercle bacilli inoculated into the soil would survive for many months.

REFERENCE

SCHALK, A. F. 1928 Jour. Am. Vet. M. Asso., **72**, 852.

FURTHER STUDIES ON FROZEN VEGETABLES[1]

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PREVIOUS WORK

In previous reports the writers have recorded results obtained in studies of tin, cardboard box (Peters' type) and glass containers of frozen peas, some of which had been inoculated with *Cl. botulinum* spores. (A. J. P. H. **22,** 473–492, May, 1932, and A. J. P. H. **23,** 700–703, July, 1933.) Botulinus toxin was found in all types of containers held at 80° to 90°F. for three to three and a half days, but no toxin was obtained from any of the containers that had been defrosted and examined immediately, or from those placed in an ice box (50 to 60°F.) and held for three days.

PRESENT WORK

The studies reported in this paper were made to determine if toxin production by *Cl. botulinum* is possible in the various types of containers of frozen peas[2] when they have been defrosted and held at ice box temperatures for a longer period than three days and if held at room temperature for a shorter period than three

[1] Food Research Division Contribution No. 234.

[2] All the containers used in both the previous and present work were prepared in Seattle, Washington, in the summer of 1930. There were from 12 to 16 ounces of peas in each container. In the preparation of the samples for freezing some of the peas were washed but not blanched, some were blanched in hot water for about two and one-half to three minutes, some were blanched in 2.5 per cent brine for about the same time, and some were put into containers and sealed under 20 inches of vacuum. After freezing they were shipped under refrigeration to Washington, D. C., and held in storage at 15° to 20°F. until examined. All were stored under the same conditions and those reported in this paper were held for two years before they were examined.

days. Accordingly the samples of frozen peas were held at the following controlled temperatures for the periods indicated: (1) 42°F. for seven days; (2) 50°F. for seven days; (3) 60°F. for three days; (4) 60°F. for six days; and (5) 80°F. for two days.

All the samples used in these studies were taken from cold storage (15° to 20°F.) and immediately placed under the desired controlled temperature. The holding period given, for example, seven days, includes both the defrosting time and the time held in the defrosted condition.

The technique employed is an improvement over that previously used and it leads to better results. Improvements were made in:

1. Preparation of materials for toxin testing.
2. Method of isolating *Cl. botulinum* cultures.

PREPARATION OF MATERIAL FOR TOXIN TESTING

Previously the peas were removed from the original container and the container washed with 50 cc. of sterile water, which was added to the sample. In the present work no water was added. The container was opened, and the peas were thoroughly crushed with a sterile spoon and then transferred to a pocket of cheesecloth over the mouth of a sterile pint jar. The peas were then thoroughly squeezed by twisting the cheesecloth. This resulted in the pressing out of 50 to 150 cc. of pea liquor. The pressed peas were discarded. All the liquor obtained was centrifuged at about 2500 r.p.m. for one hour, and the supernatant fluid was pipetted off and tested in guinea pigs. This method does not result in dilution of the liquor as did previous methods, and it is more likely to show toxin of weak strength. It is also preferred to the method of pipetting off 5 or 10 cc. from the original container because work at this laboratory indicates that there may be several types of decomposition in a mass of peas proceeding simultaneously. Some may be aerobic and some anaerobic; some may produce a high acidity and some a low acidity. When the sample is thoroughly mixed a high acidity may overbalance points of low acidity, with the apparent result of botulinus toxin production under conditions of high acidity. Thus it is important to use

the entire contents of the container in obtaining material for toxin tests.

METHOD OF ISOLATING CL. BOTULINUM CULTURES

A more satisfactory method for the isolation of *Cl. botulinum* cultures than that used in previous experiments was also devised. Formerly the contents of a container of frozen peas were defrosted and removed from the container. The original container was then washed with 50 cc. of sterile water, which was thoroughly mixed with the sample of macerated peas. A cubic centimeter of this semi-liquid, representing approximately .0025 of the entire sample, was pipetted into each of a series of tubes containing beef-heart medium, and the tubes were incubated.

In the new technique, culture isolations were made from the sediment remaining in the centrifuge tubes after the sample liquor had been centrifuged as described above. A small quantity of liquid was left in the tube to facilitate the mixing and pipetting. About 0.5 cc. of this suspended sediment was pipetted into a tube of beef-heart medium which had been previously boiled and cooled. Autoclaved vaseline was then poured on top of the medium, and the tubes were incubated at 37°C. for seven to ten days. As a check against contamination, a series of 4 or 5 tubes of beef-heart media was prepared in the same manner as described above, except that 0.5 cc. of sterile water was added instead of the 0.5 cc. of sediment from the centrifuge tubes. These control tubes were always prepared after the material. Gas production or darkening of the beef heart was accepted as presumptive evidence of growth of *Cl. botulinum*. After growth appeared in the beef-heart tubes, the contents were stirred, transferred to a centrifuge tube, and centrifuged for one hour at about 2500 r.p.m. The supernatant liquid, which was usually clear and amber in color, was pipetted off; a portion was used for the determination of the minimum lethal dose and another portion to test the thermolability of the toxin. After the minimum lethal dose had been determined the toxin was identified by the toxin-antitoxin method. The type of toxin found in this centrifuged liquid determines the type of *Cl. botulinum* culture.

RESULTS OF CULTURE IDENTIFICATIONS

A series of 60 experimental containers of frozen peas was examined by the new method. Of these containers, 30 were originally seeded with a concentrated inoculum of *Cl. botulinum* spores (100,000,000 per container) and 30 containers were uninoculated controls. *Cl. botulinum* was demonstrated in every inoculated container as well as in every uninoculated control. When using the old technique the writers were able to identify cultures of *Cl. botulinum* from only about 30 per cent of the uninoculated control containers.

TABLE 1

Number of culture isolations grouped by type and toxin titre

CONTAINERS, TOTAL NUMBER	NUMBER OF CULTURES ISOLATED										
	By type‡	By toxin titre, m.l.d.'s of toxin per cc.									
		5	50	100	200	500	700	1000	1500	2000	2200	3000
30 controls*	15 type A	2	1	2	3	1	4	2				
	15 type B						9	5	1			
30 inoculated†	0 type A											
	30 type B							1	23	1	1	4

* Controls. Original containers were not inoculated.

† Inoculated. Original containers inoculated with 100,000,000 spores of *Cl. botulinum*.

‡ Determined by type of toxin found in centrifuged liquid from media tubes.

The results obtained show that 15 of the 30 uninoculated controls contained *Cl. botulinum* type A and 15 contained type B, whereas all of the 30 inoculated containers yielded cultures of type B.[3] The minimum lethal dose of each of these toxins, based on a 1 cc. subcutaneous injection for 250 grams of guinea pig, and the types of toxin, are listed in table 1.

The results given in table 1 indicate that the toxins produced by the cultures obtained from the uninoculated peas contained fewer minimum lethal doses per cubic centimeter than did the cultures from the inoculated peas.

[3] Each inoculated container had received 100,000,000 spores of *Cl. botulinum* including 2 type A cultures and 2 type B cultures.

RESULTS OF TOXIN TESTS

I. Frozen peas held at 42°F. for seven days (includes defrosting)

Ten samples (5 in tin and 5 in glass) were originally inoculated with a concentrated inoculum of *Cl. botulinum* spores (100,000,000

TABLE 2

Frozen peas held at 50°F. for seven days (includes defrosting)

INOCULUM	TOTAL NUMBER OF CONTAINERS	PETERS' TYPE		TIN		GLASS		COOKED*	
		Number examined	Number toxic	Number examined	Number toxic	Number examined	Number toxic	Number examined	Number toxic
None	30	5	1	10	0	10	0	5	0
Concentrated†	30	5	3	10	0	10	0	5	0
Totals	60	10	4‡	20	0	20	0	10	0

* Frozen peas placed in water, brought to boiling and boiled for ten minutes, cooled and held at 50°F.

† Each container inoculated with 100,000,000 spores of *Cl. botulinum*.

‡ For details, see table 2A.

TABLE 2A

Four samples of frozen peas that became toxic while held at 50°F. for seven days (includes defrosting)

SAMPLE*	pH	TOXIN		CULTURE		COMMENTS
		Type	Minimum lethal dose per cubic centimeter	Type	Minimum lethal dose per cubic centimeter	
Controls†	5.3	A	2	B	700	Soft and very moldy
Inoculated‡	6.8	B	2	B	2200	Soft and very moldy
Inoculated‡	6.9	A	2	B	3000	Soft and very moldy
Inoculated‡	7.0	A	2	B	3000	Soft and very moldy

* All samples were packed in Peters' type of cardboard containers.

† Uninoculated.

‡ Received concentrated inoculum.

per container). None of these containers gave any evidence of toxin, although there was a slight hay-like odor indicating that spoilage had begun. The pH (colorimetric) of these 10 containers

ranged from 5.6 to 6.5. As none of the 10 containers (heavily inoculated) gave any evidence of toxin, no uninoculated or lightly inoculated samples were held at this temperature.

II. Frozen peas held at 50°F. for seven days (includes defrosting)

This series included 30 uninoculated controls and 30 that had received a concentrated inoculum of *Cl. botulinum* spores. Tables 2 and 2A give the essential details and results.

Four of the 60 samples examined (tables 2 and 2A) gave definite evidence of toxin, although the strength of the toxins was very weak (2 minimum lethal doses per cubic centimeter). One of these toxic samples was an uninoculated control, and the other three were originally inoculated with a concentrated inoculum. Three of the containers (table 2A) gave type A toxin although the cultures isolated were type B. These results were checked and re-checked.

All 4 of the toxic samples were moldy and spoiled. Most of the non-toxic samples had a sour odor; some had a putrefactive odor; and most of those in paper containers had mold growth.

III. Frozen peas held at 60°F. for three days (includes defrosting)

This series of samples included 35 containers, 20 of which were uninoculated controls, and 15 of which had originally received a concentrated inoculum. The uninoculated controls included 10 tin and 10 glass containers; the 15 inoculated glass containers included 12 without vacuum and 3 with vacuum. None of these containers gave any evidence of toxin, although the peas had a bleached appearance, and there was a slightly sour odor in the containers. The pH ranged from 4.6 to 6.7.

IV. Frozen peas held at 60°F. for six days (includes defrosting)

This series of samples was made up of 15 glass containers, each originally inoculated with a concentrated inoculum of *Cl. botulinum* spores. None of them gave any evidence of toxin. The peas had a bleached appearance and a sour odor. The pH ranged from 4.6 to 6.2.

V. Frozen peas held at 80°F. for two days (includes defrosting)

In this series 78 containers were examined, including 33 uninoculated controls, 15 that had received a dilute inoculum (less than 100 *Cl. botulinum* spores) and 30 samples that had received a concentrated inoculum. Table 3 gives the essential details.

None of the control samples (table 3) gave any evidence of toxin. The pH ranged from 4.3 to 4.8, except in the "cooked" series, where the range was from 6.1 to 6.6.

TABLE 3

Frozen peas held at 80°F. for two days (includes defrosting)

Inoculum	Total number of containers	Tin*		Glass						Peters' type* cardboard		Cooked*	
				Vacuum; blanched		No vacuum; blanched							
				Hot H_2O		Hot H_2O		2.5 per cent brine					
		Number examined	Number toxic	Number examined	Number toxic	Number examined	Number toxic	Number examined	Number toxic	Number examined	Number toxic	Number examined	Number toxic
Uninoculated	33	5	0	3	0	10	0	5	0	5	0	5	0
Dilute†	15			5	0	5	0	5	0				
Concentrated‡	30	5	0	5	1	10	0			5	4	5	1
Total	78	10	0	13	1§	25	0	10	0	10	4§	10	1§

* Blanched in hot water, packed without vacuum. Those cooked at time of examination were placed in water, brought to boiling and boiled for ten minutes, cooled and placed at 80°F.

† Less than 100 *Cl. botulinum* spores originally inoculated.

‡ About 100 million *Cl. botulinum* spores originally inoculated.

§ For details see table 3A.

No evidence of toxin was found in the samples given the dilute inoculum (less than 100 *Cl. botulinum* spores per container). The pH of 14 samples ranged from 4.5 to 4.6; one other sample had a pH of 5.8.

Six of the 30 containers originally receiving the concentrated

inoculum contained botulinus toxin. The essential details of these 6 toxic samples are shown in table 3A.

TABLE 3A

Toxic samples of frozen peas originally inoculated with a concentrated inoculum of Cl. botulinum spores and held at 80°F. for two days (includes defrosting)

SAMPLE	pH	TOXIN		CULTURE		COMMENTS
		Type	Minimum lethal doses per cubic centimeter	Type	Minimum lethal doses per cubic centimeter	
Cooked*	6.7	B	20			Haylike odor
Glass—20" vacuum	4.6	B	2			Sour odor
Peters' type container	5.0	B	1	B	1500	Soft and moldy
Peters' type container	4.8	B	10	B	1500	Soft and moldy
Peters' type container	4.8	B	5	B	1500	Soft and moldy
Peters' type container	4.7	B	1	B	1500	Soft and moldy

* Frozen peas placed in water, brought to boiling and boiled for ten minutes, cooled and held at 80°F.

DISCUSSION

Studies were made on 198 samples of frozen peas in various types of containers and included 83 uninoculated controls; 15 that had received a dilute inoculum of *Cl. botulinum* spores; and 100 that had received a concentrated inoculum (about 100,000,000 spores per container).

One of the 83 uninoculated control samples gave positive evidence of toxin. This sample was in a rectangular cardboard container (Peters' type), which had been held at 50°F. for one week. The peas were very moldy on top and very soft and probably would not have been consumed by a discriminating person. The pH was 5.3 (colorimetric) and the toxin was type A, 2 minimum lethal doses per cubic centimeter for guinea pigs. The culture isolated was type B with a titer of 700 minimum lethal doses per cubic centimeter.

None of the 15 samples receiving a dilute inoculum (less than 100 *Cl. botulinum* spores) gave any evidence of toxin.

Nine of the 100 samples receiving a concentrated inoculum of

Cl. botulinum spores (about 100,000,000) contained toxin, although in no case was the toxin very potent. Six of these toxic samples were in containers held at 80°F. for two days, and 3 were stored at 50°F. for seven days. Seven of these samples were badly spoiled and would not have been consumed. Two of the samples did not appear spoiled, but one of them had a slightly hay-like odor and the other had a slightly sour odor. Whether the contents of these containers would have been used by the housewife is questionable. However, the content in minimum lethal dose units of toxin was low, being 2 per cubic centimeter and 20 per cubic centimeter. If these peas were heated as is done in cooking, they probably would have been rendered harmless. Both of these containers had been inoculated with a concentrated inoculum (100,000,000 spores) and it is doubtful whether any commercial package would be so heavily contaminated.

Because the supply of samples was low some of these experiments did not include many containers, especially those of the Peters' type. In some cases containers that had received a concentrated inoculum of *Cl. botulinum* spores were tested first, and when no evidence of toxin was shown, tests of uninoculated controls were omitted. In some cases only glass containers were used in order to make frequent observations of signs of spoilage without opening the containers.

SUMMARY

This work was undertaken to study the possibility of toxin production in frozen peas when defrosted and held for a shorter period than three and one-half days at room temperature, and when held for a longer period at ice box temperature.

A total of 198 samples of frozen peas in various types of containers were examined as follows:

1. *83 uninoculated controls.* 33 defrosted and held at 80°F. for two days; 20 defrosted and held at 60°F. for three days; and 30 defrosted and held at 50°F. for seven days.

One of these controls, defrosted and held at 50°F. for seven days, gave positive evidence of a weak toxin.

2. *15 containers receiving a dilute inoculum.* Defrosted and

held at 80°F. for two days; and none of these samples gave any evidence of toxin.

3. *100 containers receiving a concentrated inoculum.* 30 defrosted and held at 80°F. for two days; 15 defrosted and held at 60°F. for six days; 15 defrosted and held at 60°F. for three days; 30 defrosted and held at 50°F. for seven days; and 10 defrosted and held at 42°F. for seven days.

Of these samples, 9 gave definite evidence of toxin; 6 had been held at 80°F. for two days and 3 at 50°F. for seven days.

CONCLUSION

When peas preserved by freezing are properly handled there is no danger of botulism. They should not be held at room temperature after defrosting, and left-over portions should be well refrigerated and thoroughly cooked before consumption.

BACTERICIDAL ACTION OF AZOCHLORAMID (N-N-DICHLOROAZODICARBONAMIDINE)

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A preliminary report (Schmelkes and others, 1934) on azochloramid (Schmelkes and Marks, 1934), a new chlorine compound of unusual properties, was given at the 1933 annual meeting of the Society.

Bactericidal agents such as chlorine, hypochlorites, and iodine, which derive their effectiveness from positively-charged halogens, differ from others in that they react chemically with the microorganisms and are used up in this reaction. A serious disadvantage of halogens is their tendency to react with organic matter present. It is important to reduce the extent of the reaction between the halogens and organic matter without too much impairment of bactericidal action.

Chloramine T (sodium-para-toluene-sulphonchloramide) and dichloramine T (para-toluene-sulphondichloramide) were developed some years ago from this viewpoint and excel Dakin's solution and the hypochlorites in their stability toward organic matter. Azochloramid, as shown by Guiteras and one of us (1934), shows less interaction with organic matter than any other chlorine compound used for bactericidal purposes.

As has been discussed by one of us (Schmelkes, 1933) sometime ago, this property can be correlated with the potential of an indifferent electrode in a solution of a chlorine compound. Azochloramid, in that investigation, was found to exhibit a lower potential than any chlorine compound hitherto investigated and this is quite in agreement with the fact that it reacts with and oxidizes organic matter very slowly and thus remains available for bactericidal action for long periods of time. The following

experiments indicate that this stability has been obtained without reducing the bactericidal power proportionally.

Experiments were devised to show the bactericidal power of azochloramid, chloramine T, and sodium hypochlorite on saline suspensions of washed organisms and on suspensions of organisms with the addition of varying quantities of normal horse serum.

In the experiments without organic matter, the disinfectants were diluted with physiological saline and inoculated with a suspension of washed organisms so that the final mixture contained the desired concentration of chlorine and *Staphylococcus aureus* equivalent to 0.1 cc. of a twenty-four-hour broth culture per 5 cc. The mixtures were kept in a water bath at 37.5°C. during the experiment. At given time intervals 1.5 cc. samples were dechlorinated with a drop of sodium sulphite solution of sufficient concentration to reduce completely the chlorine carried over. One-cubic centimeter portions of the dechlorinated mixture were transferred to 10 cc. of nutrient broth. This prevented any bactericidal or bacteriostatic action of the disinfectant from being carried over into the subculture medium.

Controls were set up to determine whether the sodium sulphite or the reaction products of the disinfectants with sodium sulphite were bactericidal or bacteriostatic. Washed organisms in physiological saline were carried through the experiment along with the disinfectant-organism mixtures. Portions were treated with the maximum amount of sodium sulphite used to dechlorinate the disinfectant mixture and 1-cc. portions were transferred to 10 cc. of nutrient broth. All tubes were incubated for ninety-six hours at 37.5°C. No bactericidal or bacteriostatic action was observed in these controls. In order to test the effect of the reaction products of the disinfectants and sodium sulphite, the highest concentration of each disinfectant used in the experiment was dechlorinated and inoculated with the same number of organisms used in the experiment. One-cubic centimeter portions were tranferred to nutrient broth at given intervals. No inhibition of growth was observed. All controls were positive throughout the experiments and are not noted in detail in the tables.

A summary of the results obtained in a number of these experiments is given in table 1.

To show the effect of organic matter, the experiment was repeated using a broth-culture horse-serum mixture as inoculum. The final mixture contained the desired concentration of chlorine, 10 per cent serum, and 0.1 cc. of a twenty-four-hour broth culture of *Staphylococcus aureus* per 5 cc. The sodium sulphite, and disinfectant-plus-sodium-sulphite controls were run as in the preceding experiment, except that they contained 10 per cent serum, and instead of washed organisms the same inoculum of broth culture as in the corresponding experiments. In the next experiment the amount of serum was increased to 50 per cent. The results of these experiments are shown in table 2. In these and in subsequent experiments, more time intervals and concentrations of disinfectant were run than are shown. In cases where duplication of results was obtained with several concentrations of disinfectant, only the lower concentration is shown.

TABLE 1

Action of azochloramid, chloramine T, and sodium hypochlorite on Staphylococcus aureus

Washed organisms

COMPOUND	Cl	15 MINUTES	30 MINUTES	45 MINUTES	1 HOUR	1½ HOURS	2 HOURS
	p.p.m.						
Azochloramid	1	+	+	+	+	+	+
	2	+	+	+	+	−	−
	5	+	−	−	−		
Chloramine T	1	+	+	+	+	+	−
	2	+	+	+	−	−	−
	5	−	−	−	−	−	−
Sodium hypochlorite	1	+	+	+	+	+	−
	2	+	+	−	−	−	−
	5	−	−	−	−	−	−

+, growth; −, no growth in ninety-six hours.

The controls used—Saline plus Na_2SO_3; azochloramid plus Na_2SO_3; chloramine T plus Na_2SO_3; and sodium hypochlorite Na_2SO_3—were positive at all intervals.

The data in table 1 indicate that in the absence of organic matter the three chlorinating agents are about equally effective, sodium hypochlorite being slightly better than chloramine T, and

TABLE 2

Action of azochloramid, chloramine T, and sodium hypochlorite on Staphylococcus aureus

COMPOUND	Cl	15 MINUTES	30 MINUTES	1 HOUR	1½ HOURS	2 HOURS	3 HOURS
10 per cent serum							
	p.p.m.						
Azochloramid	10	+	+	+	+	+	+
	15	+	+	+	+	−	−
	20	+	+	+	−	−	−
Chloramine T	60	+	+	+	+	+	−
	70	+	+	+	+	−	−
	80	+	+	−	−	−	−
Sodium hypochlorite	200	+	+	+	+	+	+
	250	+	+	+	+	−	−
50 per cent serum							
Azochloramid	20	+	+	+	+	−	−
	30	+	+	−	−	−	−
	40	+	−	−	−	−	−
Chloramine T	250	+	+	+	+	+	+
	300	+	+	−	−	−	−
	350	+	−	−	−	−	−
Sodium hypochlorite	800	+	+	+	+	+	−
	1,000	+	+	+	−	−	−
	1,200	+	+	−	−	−	−

+, growth; −, no growth in ninety-six hours.

The controls used—saline plus Na_2SO_3; azochloramid plus Na_2SO_3; chloramine T plus Na_2SO_3; and sodium hypochlorite plus Na_2SO_3—were positive at all intervals.

this in turn being slightly better than azochloramid. In the presence of 10 per cent horse serum, as shown in table 2, the efficiency of the disinfectants is reversed, azochloramid being about four times as effective as chloramine T and 16 times as

effective as sodium hypochlorite. The data in table 2 also show that in the presence of 50 per cent serum azochloramid is 10 times as effective as chloramine T and 40 times as effective as sodium hypochlorite.

TABLE 3

Action of azochloramid on Staphylococcus aureus

Various numbers of organisms. 50 per cent serum

NUMBER OF ORGANISMS PER CUBIC CENTIMETER	Cl	1 MINUTE	3 MINUTES	5 MINUTES	10 MINUTES	15 MINUTES	30 MINUTES	1 HOUR	1½ HOURS	24 HOURS
	p.p.m.									
39,000,000	5					+	+	+	+	+
	10					+	+	+	−	−
	20			+	+	−	−	−	−	−
	30			+	−	−	−	−	−	−
3,500,000	5					+	+	+	+	+
	10					+	+	−	−	−
	20				+	−	−	−	−	−
	30			+	+	−	−	−	−	−
300,000	5					+	+	+	+	+
	10					+	+	−	−	−
	20			+	+	−	−	−	−	−
	30		+	+	−	−	−	−	−	−
30,000	5					+	+	+	+	+
	10			+	+	+	−	−	−	−
	20	−	−	−	−	−	−	−	−	−
3,000	5			+	+	+	+	+	+	+
	10		+	+	+	+	−	−	−	−
	20	−	−	−	−	−	−	−	−	−

+, growth; −, no growth in ninety-six hours.

Saline + Na_2SO_3 controls and azochloramid + Na_2SO_3 controls were positive through twenty-four hours in all cases.

The organisms in saline used as controls in table 1 decreased in number from 925,000 at the beginning of the experiment to 8000 in two hours and to 0 in five hours. The results of the experiment are nevertheless indicative of the relative bactericidal power of azochloramid, chloramine T, and sodium hypochlorite. The organisms suspended in diluted sera used as controls in table 2,

increased from 1,800,000 at the beginning of the experiment to 14,000,000 in five hours in 10 per cent serum and to 39,000,000 in 50 per cent serum.

The influence of a number of additional factors affecting the

TABLE 4

Action of azochloramid on various strains of Staphylococcus aureus

50 per cent serum

STRAIN	Cl	15 MINUTES	30 MINUTES	45 MINUTES	60 MINUTES
	p.p.m.				
I	20	+	+	−	−
	30	+	−	−	−
	40	+	−	−	−
II	20	+	−	−	−
	30	+	−	−	−
	40	−	−	−	−
III	20	+	−	−	−
	30	−	−	−	−
IV	20	+	+	+	−
	30	+	−	−	−
	40	−	−	−	−
V	20	+	+	−	−
	30	+	−	−	−
	40	+	−	−	−
VI	20	+	−	−	−
	30	−	−	−	−
VII	20	+	+	+	−
	30	+	+	−	−
	40	+	+	−	−

+, growth; −, no growth in ninety-six hours.

Saline + Na_2SO_3 and azochloramid + Na_2SO_3 controls were positive for all strains through all time intervals.

bactericidal powers of azochloramid was investigated. All these experiments were carried out in the presence of 50 per cent horse serum and the procedure was the same as that previously described. The factors studied were the effect of various numbers

TABLE 5

Action of azochloramid on various organisms

50 per cent serum

ORGANISM	Cl	15 MINUTES	30 MINUTES	45 MINUTES	1 HOUR	11 HOURS	2 HOURS	3 HOURS	4 HOURS	24 HOURS
	p.p.m.									
Staph. aureus	20	+	+		+	+	−	−	−	−
	30	+	+		−	−	−	−	−	−
	40	+	−		−	−	−	−	−	−
Strep. hemolyticus	5	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	−
	15	+	+	+	+	+	+	+	−	−
	40	+	+	+	+	+	−	−	−	−
	100	+	+	−	−	−	−	−	−	−
Cl. Welchii (vegetative cells)	25	+	−		−	−	−	−	−	−
	50	+	−		−	−	−	−	−	−
	100	−	−		−	−	−	−	−	−
C. diphtheriae	10	+	+		+	+	+	+	+	+
	25	−	−		−	−	−	−	−	−
Pneumococcus Type I	10	+	+		+	+	+	+	+	+
	25	+	+		+	−	−	−	−	−
	50	+	+		−	−	−	−	−	−
Pneumococcus Type II	5	+	+		+	+	+	+	+	+
	10	+	+		+	+	+	+	+	−
	25	+	+		−	−	−	−	−	−
	50	+	−		−	−	−	−	−	−
Pneumococcus Type III	10	+	+		+	+	+	+	+	+
	25	+	+		+	+	+	−	−	−
	50	+	+		−	−	−	−	−	−
Esch. coli	15	+	+	+	+	+	+	+	+	+
	25	+	+	+	−	−	−	−	−	−
	60	+	+	−	−	−	−	−	−	−
Ps. pyocyanea	10	+	+	+	+	+	+	+	+	+
	25	+	+	+	−	−	−	−	−	−
	50	+	+	−	−	−	−	−	−	−
	100	+	−	−	−	−	−	−	−	−
	150	−	−	−	−	−	−	−	−	−

+, growth; −, no growth in ninety-six hours.

Saline + Na_2SO_3 controls and azochloramid + Na_2SO_3 controls were positive through twenty-four hours for each organism.

of organisms, of various Gram-positive and Gram-negative organisms, and of different strains of the same organism.

Table 3 shows that in suspensions containing from 3000 to 39,000,000 organisms per cubic centimeter there is only a slight difference in the action of azochloramid.

The resistance of seven strains of *Staphylococcus aureus* to azochloramid varied but little. The results are shown in table 4.

TABLE 6

Action of azochloramid on Staphylococcus aureus in presence of laked red blood corpuscles

50 per cent serum

LAKED RED BLOOD CORPUSCLES	Cl	15 MINUTES	30 MINUTES	45 MINUTES	1 HOUR	3 HOURS	5 HOURS
per cent	*p.p.m.*						
0	20	+	+	−	−	−	−
	30	+	−	−	−	−	−
	50	−	−	−	−	−	−
0.5	20	+	+	−	−	−	−
	30	+	+	−	−	−	−
	50	+	−	−	−	−	−
5.0	100	+	+	+	+	+	+
	125	+	+	+	−	−	−
	200	+	+	−	−	−	−
	250	+	−	−	−	−	−

+, growth; −, no growth in 96 hours.

Saline + Na_2SO_3 controls and azochloramid + Na_2SO_3 controls were positive through twenty-four hours in the presence of 0, 0.5, and 5.0 per cent laked red blood corpuscles.

The effect of azochloramid on various organisms, including both Gram-positive and Gram-negative species as shown in table 5 indicates that while the resistance of the organisms varies, all are killed by low concentrations.

In duplicating these experiments fluctuations were noted which were finally traced to the different lots of serum. It was found that the variations were related to the depth of color of the serum which was apparently caused by the presence of small amounts of hemolyzed red blood corpuscles. In order to study the effect

of hemolyzed red blood corpuscles, 0.5 and 5.0 per cent of laked corpuscles were added to straw-colored serum before inoculation. The efficiency of azochloramid, as shown in table 6, is impaired by the presence of laked red blood corpuscles.

SUMMARY

It has been shown that azochloramid possesses the property of killing microörganisms in the presence of organic matter such as serum. In this respect it excels all other chlorine compounds. Only actively reducing organic matter such as hemoglobin is an exception.

Like other chlorine compounds, azochloramid shows very little specific action and its solutions can be used efficiently for bactericidal purposes on various kinds of microörganisms.

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PRODUCTS FROM THE FERMENTATION OF GLUCOSE AND ARABINOSE BY BUTYRIC ACID ANAEROBES[1]

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As part of a comprehensive survey of a large number of anaerobic, butyric-acid forming bacteria and related types, the fermentation products from the decomposition of glucose and arabinose were investigated. A bacteriological study of part of the group has previously been reported by McCoy, et al. (1930).

Butyric acid and butyl alcohol (Pasteur (1861), Fitz (1882), Gruber (1887), Botkin (1892), Beijerinck (1893), Grimbert (1893), Winogradsky (1902)) have long been distinguished and accepted as fermentation products characteristic of at least some of the group. Beijerinck listed four distinct types of fermentation, while Bredemann (1909) recognized only one with varying amounts of lower-boiling alcohols and acids. Acetone (Schardinger (1905), Breaudat (1906)) and isopropyl alcohol (Pringsheim (1906), Morikawa and Prescott (1927)) were later recognized as commonly-occurring fermentation products. In addition, certain other compounds such as isobutyl alcohol (Grimbert (1893), Folpmers (1920), Canonici (1932)) and amyl alcohol (Perdrix (1891)) have been reported, but their occurrence as direct products of carbohydrate decomposition is still uncertain.

EXPERIMENTAL

Cultures

The cultures investigated are designated as strains 1 to 5 inclusive, 7 to 26, 28 to 33, 36 to 44 and 46 to 57. Of these the cul-

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tures numbered 1 to 33 are those reported by McCoy, et al. (1930) and the numbers correspond to their designation. In addition to these, new isolations include strains 36, 40, 53, 54, 56 and 57. Cultures from other sources not previously reported are:

STRAIN	DESCRIPTION	SOURCE
37	F. B. Fernbach	Collection of Sir Frederick Andrewes
38	F. B. BB	
39	*B. butylicus* Fitz	
42	Pink BY	
43	German Maize	
44	Feeble pink white	
46	*B. butylicus*	
A	*B. butylicus* x 160	
B	*B. butylicus* Bakonyi	
41	*Cl. felsineum*	Collection of Dr. A. J. Kluyver
H	*B. butylicus* (Hall BY Cat. No. 3625)	American Type Culture Collection
T	*B. butylicus* (Thaysen BY Cat. No. 4259)	
51	*Cl. multifermentans* (Cat. No. 3538)	
52	*Cl. pectinovorum* (Cat. No. 859)	

According to cultural characteristics the organisms studied may be grouped as follows: Strains 1 to 7 inclusive are of the *Cl. Pasteurianum* type, which does not attack starch. The *B. saccharobutyricus* type, which partially hydrolyzes starch includes strains 8 to 28, 36, 44, 46 and 57. Strains 29 to 33 are starch-fermenting plectridia, while strains 37 to 39 are of the *B. butylicus* type. The starch-fermenting butyl alcohol producers include strains A, B, H and T, which are of the *Cl. acetobutylicum* type, strain 40, which is an unknown pectin-fermenting type, strain 41, *Cl. felsineum*, and strains 42 and 43, *Cl. roseum*.

The inoculum used in the fermentations was prepared as follows: 10 cc. of 6 per cent corn mash in a tube was inoculated with a spore culture in soil. This tube was pasteurized in boiling water for one minute, cooled and incubated at 37°C. When this culture showed vigorous activity, as it did after a period varying from eighteen to twenty-four hours, it was transferred to duplicate tubes of 3 per cent glucose in double-strength yeast water. One of these tubes was used as the inoculum at an age of eighteen to twenty-four hours.

Analytical methods

The determinations of glucose and arabinose were carried out according to the method of Stiles, Peterson and Fred (1926). For the determination of titrable acidity, 10 cc. of the culture was brought to a boil to drive off carbon dioxide, cooled and titrated with 0.1 N sodium hydroxide to the phenolphthalein endpoint.

Butyl, ethyl and isopropyl alcohols and acetone were determined on distillates of the fermented culture. For this purpose 15 cc. of the culture was placed in a 38 x 200 mm. Pyrex test tube, made alkaline with 2 cc. of N sodium hydroxide and 5 to 8 cc. was distilled off and collected under carbon-dioxide-free water in a 50 cc. volumetric flask. The distillate was then made up to 50 cc. with carbon-dioxide-free water. To prevent bumping during the distillation a few glass beads were added, and to reduce foaming small amounts of sodium sulphate and magnesium sulphate were used. Butyl and ethyl alcohols were determined on an aliquot by the method of Johnson (1932). Acetone was determined by a modification of Goodwin's method (1920).

Isopropyl alcohol was determined according to a procedure for the dichromate method developed in these laboratories by R. J. Allgeier and E. L. Tatum as follows: A sample is placed in a 38 x 200 mm. Pyrex test tube together with 10 cc. of 1.5 N potassium dichromate in 5 N sulphuric acid. The volume is made up to exactly 25 cc. with distilled water and three glass beads are added. A rubber stopper is then wired in and the tube is placed in a boiling water bath for exactly three minutes, after which it is removed and cooled in running water. The sample is then distilled from the tube, the distillate being collected under the surface of 15 cc. of N sodium hydroxide in a 125 cc. Erlenmeyer flask. When about half of the liquid has been distilled off, 5 cc. of 0.2 N iodine is added to the distillate by rapid dropping from a pipette. After standing for five to ten minutes the sample so treated is made acid with 20 cc. of N sulphuric acid and the liberated iodine is titrated with 0.1 N sodium thiosulphate from a microburette. A blank for the purpose of standardizing the iodine solution is also titrated, and each cc. of 0.1 N iodine used by the sample is equivalent to 0.967 mgm. of acetone. Of the

isopropyl alcohol originally present in the sample, 85 per cent is recovered in the distillate as acetone, while 96 per cent of the original acetone is recovered as such.

For the determination of butylene glycol the extraction method as used by Fulmer, et al. (1933) was employed, except that, instead of drying the extract for two days in a desiccator, it was dried to constant weight, which required a period of about two weeks. Acetylmethylcarbinol was determined by a modification of the method employed by Wilson, et al. (1927).

Selection of medium

A group of representative cultures was cultivated on four different media for the purpose of choosing the most suitable. The media were:

1. Single strength yeast water—the clear water extract of fresh, starch-free yeast, 100 grams per liter of tap water.
2. Double-strength yeast water—the clear water extract of fresh, starch-free yeast, 200 grams per liter of tap water.
3. A mineral-salts peptone medium (McCoy, et al. (1930)).
4. Mineral-salts peptone medium to which had been added 25 cc. of potato juice per liter.

To each of the above, 3 per cent of glucose was added.

The cultures (11, 14, 16, 22, 32, 36 and 40) chosen for this preliminary study were started in corn mash and after eighteen hours were transferred to fresh tubes of the same medium. This procedure was repeated for a third transfer.

It was found that in the plain mineral-salts peptone medium all cultures died out after the third transfer, and in that containing potato juice only two cultures were still active. In single-strength yeast water five cultures were still active, as was the case in double-strength yeast water. However, the double-strength yeast water medium appeared to be the most generally favorable since in it those cultures which did not appear active after twenty-four hours became so after another day of incubation.

In order to study further the effect of variations in the medium, it was attempted to ferment 5 per cent of glucose in media of various compositions as follows:

Number 1. Single-strength yeast water.
Number 2. Double-strength yeast water.
Number 3. Quadruple-strength yeast water.
Number 4. Double-strength yeast water + 0.5 per cent NaH_2PO_4.
Number 5. Double-strength yeast water + 0.5 per cent $(NH_4)_2SO_4$.
Number 6. Double-strength yeast water + 0.5 per cent peptone.

All media were adjusted to pH 7.0.

These media, each with 5 per cent of glucose, were inoculated with a selected group of cultures. One series of tubes inoculated with cultures 8, 11, 14, 16, 22, 32, 36, 40, 41 and 42 was incubated at 37°C. for a period of five days, while another series inoculated with cultures 8, 22, 32, 36, 40, 41 and 42 was incubated at 30°C. for six days. The cultures were then analyzed for residual glucose.

While in some cases a modified medium appeared to be preferable, in no case did all cultures respond to a single medium. Destruction of more than four-fifths of the sugar was obtained in 7 per cent of the cases with medium number 1, 59 per cent with medium 2, 65 per cent with medium 3, 39 per cent with medium 4, 35 per cent with medium 5 and 52 per cent with medium 6. From these results it is apparent that the most suitable basic medium is either medium 2 or 3 (double or quadruple-strength yeast water), and also that complete fermentation of a 5 per cent glucose concentration could not be expected. Therefore, on the basis of these results and in order to eliminate any effects due to sugar concentration, it was decided to reduce the glucose content. The basic medium finally chosen was a double-strength yeast water containing 2.5 per cent glucose and with the pH adjusted to 7.0.

Products of fermentation

Of the medium chosen, 40 cc. portions were placed in calibrated 1 x 8 inch tubes which were plugged with cotton and sterilized for forty-five minutes at 15 pounds pressure. Immediately after cooling, a 2.5 per cent inoculum was added to duplicate tubes and

these were incubated at 37°C. After four days of incubation the tubes were analyzed for residual glucose, acidity, butyl, ethyl and isopropyl alcohols and acetone.

On the basis of the analytical data the cultures have been grouped as will be brought out in the following discussion.

Group 1. Cultures producing isopropyl alcohol. Data for the cultures producing isopropyl alcohol are recorded in table 1.

TABLE 1

Fermentations characterized by production of isopropyl alcohol

CULTURE NUMBER	GLUCOSE FERMENTED	ACIDITY 0.1 N NaOH IN 10 CC.	NEUTRAL VOLATILE PRODUCTS (BASED ON GLUCOSE FERMENTED)				
			Butyl alcohol	Ethyl alcohol	Isopropyl alcohol	Acetone	Total
Group I							
	per cent	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
21	94.9	3.95	19.2	2.5	4.7	0.5	26.9
22	94.9	3.70	22.2	2.2	4.4	0.7	29.5
36	94.1	3.75	19.3	2.3	5.2	0.9	27.7
18	78.0	5.30	17.8	2.1	4.5	0.6	25.0
24	75.1	4.85	16.6	2.8	6.1	0.8	26.3
Group II							
20	94.8	4.30	15.7	2.4	3.2	1.6	22.9
30	93.1	3.65	21.0	1.7	3.8	1.3	27.8
46	94.5	3.30	17.1	2.4	3.8	3.7	27.0
Group III							
*	40.5–27.2	5.35–5.20	11.2–0.5	1.8–1.3	1.3–0.3	0.5–0.2	14.8–2.4

* Includes cultures 3, 4 and 57.

Strain 57 is included on the basis of an earlier experiment in which isopropyl alcohol to the extent of 21.5 per cent of the neutral products was found. In addition, strains 3 and 4, which also gave low results, may only be tentatively grouped as isopropyl-alcohol-producing types. It will be noted that of the eight definite isopropyl types reported, strains 18, 21, 22, 24 and 36 produce very little acetone while strains 20, 30 and 46 produce acetone and isopropyl alcohol in varying proportions.

Group II. Cultures producing acetone. This group contains the majority of the cultures studied and includes those in which the products, butyl and ethyl alcohols and acetone, are found. Table 2 presents data for those cultures which transform 20 per cent or more of the carbohydrate to neutral volatile products, while data for the remainder are presented in table 3. From these cultures butyl alcohol is the chief product, acetone is second in importance and ethyl alcohol is least important. It appears from examination of the data that certain bacteria of this group produce neutral volatile products amounting to about 30 per cent of the fermented glucose (strains 10, 11, 13, 14, 16 and perhaps 8). On the other hand strains 7, 12, 15, 17, 19, 25, 38, 39 and 41 produce about 20 per cent of neutral volatile products on the basis of the glucose fermented. Of the latter group certain strains (15, 17, 19, 25, 41) produce abnormally high final acidity and this may explain the reduced production of neutral volatile products. In the remaining four cultures, 7, 12, 38 and 39, some other compound which was not detected by analysis may have been produced, or there may have been an unusually high production of gas.

The remaining cultures, which are tabulated in table 3, include those which convert a low percentage of the carbohydrate to neutral volatile products, and a group which may be called "poor fermenters," which utilize less than 30 per cent of the glucose.

It should be kept in mind that modification of the medium may serve to improve the fermentations produced by some of the cultures, but for purposes of comparison it is essential that the same medium be used in all fermentations. For instance, strains A, B, C, H and T which, on the basis of cultural behavior, are strains of *Cl. acetobutylicum*, the industrial butyl alcohol organism, give poor fermentations on the medium used, as may be seen from table 4. It is well known that this organism ferments corn mash vigorously and completely, and will ferment glucose well in a synthetic, mineral-salts peptone medium. However, the observation that this organism shows poor fermentation on a yeast water medium serves as a useful means of comparison with related organisms.

Group III. Cultures producing ethyl alcohol and butylene glycol.

TABLE 2

Acetone-producing types which convert over 20 per cent of the glucose to neutral volatile products

CULTURE NUMBER	GLUCOSE FERMENTED	ACIDITY 0.1 N NaOH IN 10 CC.	NEUTRAL VOLATILE PRODUCTS (BASED ON GLUCOSE FERMENTED)			
			Butyl alcohol	Ethyl alcohol	Acetone	Total
	per cent	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*
12	95.4	3.75	15.8	1.3	2.5	19.6
38	95.0	3.20	17.0	1.6	2.8	21.4
8	94.7	4.00	19.2	2.5	2.9	24.6
7	93.7	3.50	15.9	2.1	3.2	21.3
10	93.5	3.75	22.4	2.5	4.1	29.0
11	93.5	3.50	23.6	2.8	3.8	30.2
16	93.3	3.30	20.8	2.5	6.2	29.5
39	93.2	3.30	16.6	1.2	3.5	21.3
13	92.2	3.70	24.5	2.8	4.6	31.9
14	92.2	3.50	25.6	2.0	3.6	31.2
41	79.6	6.70	14.4	1.8	4.4	20.6
19	69.8	5.25	14.5	3.0	3.2	20.8
17	61.5	6.00	17.8	3.2	0.8	21.8
15	59.9	5.20	14.4	2.8	2.9	20.1
25	53.9	4.35	17.8	1.7	1.2	20.8

TABLE 3

Acetone-producing types which give low yields of neutral products from glucose

CULTURE NUMBER	GLUCOSE FERMENTED	ACIDITY 0.1 N NaOH IN 10 CC.	NEUTRAL VOLATILE PRODUCTS (BASED ON GLUCOSE FERMENTED			
			Butyl alcohol	Ethyl alcohol	Acetone	Total
	per cent	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*
5	62.2	9.25	5.1	1.2	0.7	7.0
1	58.6	9.30	7.3	1.3	0.7	9.3
2	57.6	9.80	7.7	1.9	0.6	10.2
42	56.5	9.85	4.6	3.5	1.5	9.6
43	46.5	9.05	2.8	3.1	1.0	6.9
23	39.6	7.00	7.0	4.0	0.5	11.5
28	37.2	9.40	4.6	3.6	2.0	10.2
37	33.0	6.60	1.7	2.4	0.8	4.9
55	32.2	6.05	9.1	1.9	1.0	12.1
*	26.2–2.7	6.45–3.65	14.8–0.3	11.5–2.4	4.9–0.5	31.2–3.5

* Includes cultures 9, 26, 29, 31, 32, 33, 40, 44, 51 and 52.

A third distinct group which differs markedly from those already described is presented in table 5. These organisms are not strictly anaerobic but are facultative and cause pellicle formation in the yeast water medium. According to the analytical methods employed the presence of ethyl alcohol and acetone was indicated. However, since oxidation with acid potassium dichromate caused

TABLE 4

Behavior of various strains of Cl. acetobutylicum on yeast water medium

CULTURE NUMBER	GLUCOSE FERMENTED	ACIDITY 0.1 N NaOH IN 10 CC.	NEUTRAL VOLATILE PRODUCTS (BASED ON GLUCOSE FERMENTED)			
			Butyl alcohol	Ethyl alcohol	Acetone	Total
	per cent	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*
A	43.2	8.25	3.2	3.8	1.2	8.2
B	46.8	9.45	3.4	2.1	1.2	6.7
H	41.5	9.40	1.7	2.2	1.0	4.9
T	42.3	9.25	1.8	2.2	1.3	5.3
C*	55.5	9.70	4.6	4.1	2.0	10.7
B*	50.8	8.80	6.5	4.5	2.7	13.6

* These determinations were made on 4-liter fermentations of the same medium. Culture C is an authentic strain of *Cl. acetobutylicum*.

TABLE 5

Butylene glycol types

CULTURE NUMBER	GLUCOSE FERMENTED	ACIDITY 0.1 N NaOH IN 10 cc.	PRODUCTS (BASED ON GLUCOSE)			
			Butylene glycol	Acetylmethyl-carbinol	Ethyl alcohol	Total
	per cent	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*
53	89.3	2.30	6.0	3.8	14.7	24.5
56	89.7	2.35	7.1	6.9	18.0	32.0
54	20.2	2.80			24.7	24.7

the acetone figure to disappear, it was concluded that this product could not be present.

A 20-liter fermentation was then set up, using strain 53, to determine the character of the products formed. The fermented material was repeatedly distilled in order to concentrate the volatile constituents. The concentrated material (about 600 cc.) was then fractionated between 77° and 99°C. through a col-

umn. The fractions were then dried overnight with anhydrous potassium carbonate and the top layers obtained were combined and refractionated.

Fractions were collected as follows: I. 76° to 76.5°C.—5 cc. II. 76.5° to 77°C.—9 cc. III. 77° to 77.3°C.—14 cc. IV. 77.3° to 79°C.—10 cc.

Fraction I gave the iodoform reaction, but no precipitate was obtained with phenylhydrazine. The derivative made by treatment by 3, 5-dinitrobenzoyl chloride had a melting point of 89° to 91°C. The derivative made with ethyl alcohol melted at 91°C. and a mixture of the two melted at 91° to 91.5°C. The refractive index was determined on the two fractions, I and III, at room temperature, and was found to be 1.3610 and 1.3600 respectively. The value reported for ethyl alcohol at 25°C. is 1.360.

To test for the presence of acetylmethylcarbinol the method of Lemoigne as modified by Kluyver, et al. (1925) was used, in which the compound is converted to the insoluble red nickel salt of dimethylglyoxime. This test was found to be negative when applied to a composite sample of the various fractions, but was positive with the residue from the first fractionation. The presence of 2, 3-butylene glycol was demonstrated in the residue from the original distillation after first removing the acetylmethylcarbinol by oxidation with ferric chloride and distillation, after which the glycol remaining in the residue was oxidized by bromine to diacetyl and the latter was converted to the characteristic red salt.

The production of ethyl alcohol, 2, 3-butylene glycol and acetylmethylcarbinol is characteristic of a species which Donker (1926) has called *Aerobacillus polymyxa*. The organisms reported in table 5 are similar in cultural and metabolic behavior to the species described by Donker, and are evidently related to it. Strain 54 has repeatedly exhibited poor fermentation of glucose, but on the basis of cultural characteristics is included with the more vigorous types, strains 53 and 56. However, because of the weak fermentation, no conclusive analytical results on butylene glycol and acetylmethylcarbinol could be obtained for strain 54.

For these fermentations with strains 53, 54 and 56, 500 cc.

flasks containing 300 cc. of medium were used; except for size these experiments were carried out in exactly the same way as the other fermentations. The increased volume of medium was necessary to supply samples for the butylene glycol and acetylmethylcarbinol determinations.

Arabinose fermentation

On the basis of their behavior on glucose, a series of 20 of the cultures were selected for study with respect to their products

TABLE 6

Products from the fermentation of arabinose

CULTURE NUMBER	ARABINOSE FERMENTED	ACIDITY 0.1 N NaOH IN 10 CC.	NEUTRAL VOLATILE PRODUCTS (BASED ON ARABINOSE FERMENTED)				
			Butyl alcohol	Ethyl alcohol	Isopropyl alcohol	Acetone	Total
	per cent	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
40	95.8	2.9	13.7	1.5	2.2	5.2	22.6
20	44.3	4.9	17.8	1.4	1.7	0.6	21.5
57	30.9	5.1	1.4	1.6	0.6	0.6	4.2
42	95.5	6.8	10.7	3.5		7.9	22.1
8	44.1	4.7	9.9	1.1		0.6	11.6
12	40.8	4.9	13.4	1.2		1.0	15.6
28	37.6	5.2	5.5	2.9		0.5	8.9
7	36.1	4.8	13.0	0.4		0.8	14.2
13	35.3	5.0	14.0	0.5		0.7	15.2
25	33.3	5.3	5.5	2.9		0.5	8.9
19	30.0	4.9	7.1	2.4		0.9	10.4
C	35.3	11.4	1.3	1.7		0.8	3.8
*	29.1-8.5	5.9-2.8	11.9-1.4	5.0-1.3	1.0-0.0	1.1-0.2	14.4-4.0

* Includes cultures 15, 16, 17, 21, 24, 26, 32, 44 and 46.

from the fermentation of *l*-arabinose. The sugar was sterilized separately in aqueous solution and added aseptically to the yeast water to produce a medium of 2.5 per cent arabinose. The *l*-arabinose used had a specific rotation of 106°C. Except for the separate sterilization of the sugar solution exactly the same procedure was employed for the arabinose fermentations as for the glucose fermentations.

Table 6 shows that, in general, the fermentation of arabinose

was poorer than that of glucose, although strains 40 and 42 gave better results on this substrate. Van der Lek (1930) found that with some members of the group poor fermentation of arabinose was associated with a high proportion of butyl alcohol and butyric acid, and he ascribed the poor fermentation to the high proportion of butyric acid present. On the other hand, Johnson, et al. (1931) found that in the arabinose fermentation with *Cl. acetobutylicum* the proportion of oxidized products, acetone and acetic acid, increased markedly at the expense of the reduced products, butyl alcohol and butyric acid. In the present experiments a strain of *Cl. acetobutylicum*, designated as culture C, was found to give very poor fermentation, with a very high final acidity and a very low production of neutral volatile products. In the distribution of neutral volatile products from this organism, the butyl alcohol and acetone were depressed with an increase in the proportion of ethyl alcohol, as was found also to be the case in the glucose fermentation.

It will be noted that in general the acid production was higher in the arabinose fermentation than in the glucose fermentation, and that the increased acidity accompanies a lower yield of neutral volatile products. Even when good fermentations were obtained, as with strains 40 and 42, the production of neutral volatile products was lower than was obtained in good fermentations of glucose. In the fermentation with strain 42 this condition is undoubtedly due to the high acid formation, while with strain 40 it may be due to high production of gas or of some other product which was not determined in these experiments.

DISCUSSION

It is especially notable that decreased yields of neutral products are in general associated with high final acidity. This is to be expected if the neutral products are assumed to arise from the reduction of corresponding acids (Peterson and Fred (1932)). When the mechanism is interrupted or the conditions become unsuitable for such a reduction, it may be expected that acids will accumulate with consequent inhibition of further activity. If the acids were neutralized fermentation of glucose would probably

continue, but the end products would then consist largely of acids with few or no neutral products.

The occurrence of isopropyl alcohol among the fermentation products of this group of bacteria seems to be a variable characteristic. For instance, strain 40, when cultivated on glucose which it fermented poorly, was not found to produce isopropyl alcohol, but, when cultivated on arabinose which was vigorously fermented, isopropyl alcohol was obtained as a product. A somewhat similar result is reported by Van der Lek (1930) who found that in one fermentation with *Cl. felsineum* large amounts of isopropyl alcohol were obtained, while in a second experiment a small amount of this product was obtained, and in a series of subsequent experiments no evidence of the presence of isopropyl alcohol could be found. It therefore seems probable that some of the organisms which are tabulated among the poor fermenters of the acetone-producing group may, under more favorable conditions of fermentation, prove to be capable of producing isopropyl alcohol.

The behavior of *Cl. acetobutylicum* (table 4), the industrial butyl alcohol organism, is unusual, but can be explained. It will be noted that the distribution of neutral volatile products is different from that normally obtained, the fermentation is incomplete and there is a high final acidity. The results here reported show a much higher proportion of ethyl alcohol than is normal in the distribution of neutral products or "solvents" obtained with a suitable substrate. However, on the basis of carbohydrate decomposed, the weight of ethyl alcohol obtained in these fermentations is approximately the same as that obtained in the normal fermentation, while the production of butyl alcohol as well as of acetone is much reduced. These facts lead to the conclusion that ethyl alcohol is more readily formed by the organism from some central intermediate product. If that intermediate is acetaldehyde as has been suggested by several investigators (Peterson and Fred (1932)), such a conclusion obviously has a sound foundation, since the formation of butyl alcohol and acetone requires condensation reactions while ethyl alcohol may be formed through the operation of a simple Cannizzaro reaction (Peterson and Fred (1932)).

Comparison of the isopropyl alcohol-producing cultures with corresponding acetone producers brings out an interesting relationship. In the case of isopropyl-alcohol producers the ratio of butyl alcohol to total neutral volatile products is lower than in the case of acetone producers, indicating the manner in which oxidized products are balanced against reduced products. Since isopropyl alcohol corresponds to acetone in its reduced form, the production of that compound results in a decreased production of butyl alcohol, which is the most reduced of all the products.

SUMMARY

1. Fifty-two butyric acid-forming bacteria and related cultures have been studied with respect to fermentation of glucose and arabinose and production of neutral products.

2. After comparison of several media a yeast water medium containing 2.5 per cent of glucose was selected as the most generally favorable.

3. Low yields of neutral products are associated with high concentrations of acid.

4. Lower yields of butyl alcohol are obtained when isopropyl alcohol replaces acetone as a product.

5. The formation of ethyl alcohol in fermentations proceeds more readily than does the formation of acetone and butyl alcohol.

6. Arabinose is only poorly fermented by most of the cultures with production of high acidity and, generally, smaller amounts of neutral products.

7. On the basis of glucose fermented and products formed, the cultures may be grouped as follows:

I. Isopropyl alcohol producers: 11 strains.
   A. Cultures producing isopropyl alcohol and little or no acetone: 5 strains.
   B. Cultures producing both isopropyl alcohol and acetone: 3 strains.
   C. Poor fermenters: 3 strains.

II. Acetone producers: 38 strains.
   A. Cultures converting over 20 per cent of the glucose to neutral volatile products: 15 strains.

B. Cultures fermenting more than 30 per cent of the glucose but producing less than 20 per cent of neutral volatile products: 13 strains.

C. Poor fermenters; cultures fermenting less than 30 per cent of the glucose: 10 strains.

III. Polymyxa type: 3 strains.

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STUDIES ON THE ESCHERICHIA-AEROBACTER INTERMEDIATES[1]

I. CULTURAL CHARACTERISTICS

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The existence of a group of Gram-negative, non-sporogenous, lactose-fermenting, aerobic bacilli, which is apparently intermediate with respect to the Escherichia and Aerobacter genera, has been reported frequently during the past decade. This group resembles the Escherichia genus in that it is methyl-red positive, Voges-Proskauer negative and produces either a colon or a colon-like type of colony on eosin-methylene blue agar. In other respects it resembles the Aerobacter genus, in that many of the cultures utilize citrate as a sole source of carbon and ferment cellobiose, while some are citrate negative and cellobiose positive, and a few are citrate positive and cellobiose negative. No single differential test has been devised which will distinguish all members of this "intermediate" group from both the Escherichia and Aerobacter genera. The production of hydrogen sulphide will serve, however, as a differential characteristic for some of the cultures.

Studies of the cultural characteristics of the Escherichia-Aerobacter "intermediates" have been limited in the past chiefly to the common tests employed to separate the Escherichia and Aerobacter genera for the purpose of estimating the sanitary quality of water. With the exception of the studies of Koser (1924) and

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Werkman and Gillen (1932) on citrate-positive cultures, no detailed investigation has been made concerning the characteristics of this group of organisms.

During the summer of 1931, one of us (R. P. T.) isolated 18 methyl-red positive and Voges-Proskauer negative cultures, which were tentatively classified as Escherichia-Aerobacter "intermediates." Of them, 11 utilized citrate and fermented cellobiose, 6 were citrate negative and cellobiose positive, and 1 was citrate positive and cellobiose negative. With this variety of cultures available, especially the cellobiose-positive and citrate-negative group, a detailed investigation of their characteristics seemed to be in order because of Werkman and Gillen's (1932) proposal to create a new genus, Citrobacter, to include "the coli-aerogenes intermediates utilizing the citrate radical, methyl-red positive or weakly so and not producing acetoin from glycerol or glucose or in traces only from the latter." This paper presents the results obtained in such a detailed study and calls attention to the heterogeneity of the group.

METHODS AND MATERIALS

Cultures. Twenty-nine cultures were used in this study. Strains M8B, V5, V16A, V22A, V26 and V27 were kindly sent us by Dr. Koser from his collection of "coli-aerogenes intermediates" isolated from soil; M8BK, 24R, 55 and Fr (*Freundii*) were made available through the courtesy of Dr. Werkman from the group studied by Werkman and Gillen (1932); the remainder were isolated in this laboratory from eosin methylene-blue plates which had been used in the Rochester Health Bureau Laboratories for routine water analysis. Our own cultures were replated several times for colony isolation from eosin methylene-blue agar, and the entire group was plated on nutrient, eosin methylene-blue and Simmons' citrate agars to give assurance of their purity.

All of the cultures studied were Gram-negative, non-sporogenous, glucose and lactose fermenting, aerobic bacilli, which gave positive methyl-red and negative Voges-Proskauer tests in Clark and Lubs' medium (1915). All cultures, however, did not give rise to the same type of colony on eosin methylene-blue

agar. Some produced colonies of the typical colon type, i.e. dark purplish-black in color with a metallic sheen; others gave an atypical colon type of growth, dark reddish-purple without sheen, and still others yielded colonies that were bluish-purple without sheen.

Media. Most of the media employed are listed in table 1. It should be noted that several media were used in some instances for the determination of a single characteristic. The media used for studying fermentation reactions were prepared by adding the required carbohydrate, to yield a concentration of 0.5 or 1.0 per cent, to a nutrient broth, composed of 0.5 per cent peptone (Bacto) and 0.3 per cent meat extract (Bacto), which had been adjusted to pH 7.2. Bromcresol purple served as an indicator. Sterilization was accomplished by autoclaving at 15 pounds pressure for fifteen minutes, or by filtration, when the nature of the particular carbohydrate made this necessary. With the exception of Baker's soluble starch, all the carbohydrates employed were either Pfanstiehl or Eastman products. The peptone iron, lead acetate and Simmons' citrate agars and tryptophane broth were prepared from Difco dehydrated products.

Methods. The experimental methods are summarized in table 1. It will be noted that, in certain instances, the incubation period was prolonged in order to detect delayed reactions. In determining the utilization of the citrate radical in Koser's media, only glassware which had been cleaned in dichromate sulphuric-acid solution and rinsed several times with distilled water to remove all organic matter was employed. Inoculations were made with a needle from broth cultures twelve to eighteen hours old. Any culture which grew in these media was carried through at least two serial transfers. Two methods were employed to determine the action on starch. Starch agar plates were inoculated by surface streaking and flooded with iodine solution after incubation for from one to seven days. Starch broth containing bromcresol purple was used to detect acid production.

During the course of this study, all the cultures were repeatedly tested for motility, the methyl red and Voges-Proskauer reactions, the production of indol and hydrogen sulphide, the utilization of

TABLE 1

Summary of experimental methods

TEST	MEDIUM	INCUBATION		REAGENT
		Temperature	Test Period*	
		°C.		
Motility	(1) 2 per cent peptone water	37	8–18 hours	
	(2) 0.5 per cent nutrient agar	37	1– 4 days	
Ammonia production	2 per cent peptone water	37	18–24 hours	Nessler's solution
Catalase production	2 per cent peptone water	37	18–24 hours	3 per cent H_2O_2
Nitrate reduction	0.1 per cent KNO_3 in 2 per cent peptone water	37	18–24 hours	Sulfanilic acid, dimethyl-α-naphthylamine
Indol production	(1) 2 per cent peptone water	37	1– 5 days	Ether extraction, Ehrlich's reagent
	(2) 1 per cent tryptophane broth	37	1– 5 days	
Methyl red test	Clark and Lubs' (1915)	37	1– 5 days	Methyl red
Voges-Proskauer test	Clark and Lubs' (1915)	37	1– 5 days	Creatine and 33 per cent KOH
H_2S production	(1) Lead acetate agar	37 and room	1– 6 days	
	(2) Peptone iron agar	37 and room	1– 6 days	
Gelatin liquefaction	Nutrient gelatin	Room	1–30 days	
Citrate utilization	(1) Koser's (1923)†	37	1– 5 days	
	(2) Koser's (1924)†	37	1– 5 days	
	(3) Simmons' citrate agar	37	1– 7 days	
Reaction in litmus milk	Litmus milk	37	1–20 days	
Carbohydrate fermentation	0.5 or 1.0 per cent carbohydrate in nutrient broth	37	1–14 days	

* Tested or observed daily.

† Carried through at least two serial transfers.

citrate and the fermentation of glucose, cellobiose, lactose, sucrose, *a*-methylglucoside, salicin, dulcitol, glycerol, dextrin and starch. Furthermore, six months after the major portion of the study had been completed, all the cultures were again plated on eosin methylene-blue agar and from 3 to 6 sub-cultures isolated from each parent culture. The characteristics just mentioned, with the exception of starch fermentation, were then redetermined for each of the sub-cultures.

TABLE 2

Characteristics common to all cultures

MORPHOLOGY AND BIOCHEMICAL TESTS	CARBOHYDRATES FERMENTED WITH ACID AND GAS	CARBOHYDRATES NOT FERMENTED
Bacilli	*l*-Arabinose	*l*-Arabitol
Non-sporogenous	Glucose	Erythritol
Gram-negative	Galactose	Glycogen
Aerobic	*d*-Levulose	Inulin
Gelatin not liquefied	*d*-Mannose	Adonitol (except 748)
Nitrates reduced	Rhamnose	Inositol (except Fr)
Ammonia produced	Lactose	
Catalase produced	Maltose	
Methyl-Red positive	Trehalose	
Voges-Proskauer negative	Glycerol	
	Mannitol	
	d-Arabinose (except XI)	
	l-Xylose (except XI)	
	Gentiobiose (except XI)	
	Melibiose (except 418)	

RESULTS

The results of the detailed study of the cultural characteristics of the 29 Escherichia-Aerobacter "intermediate" cultures are presented below, for the most part in tables 2 through 7. All strains were found to possess certain features in common, as shown in table 2, where certain fermentative reactions are also recorded, because all but one of the cultures in each instance behaved similarly. All were methyl-red positive and Voges-Proskauer negative, and all fermented glucose, lactose and glycerol with the production of acid and gas.

These cultures differed significantly in other characteristics, as

TABLE 3

Differential characteristics of all Escherichia-Aerobacter "intermediates" studied•

CULTURE	601	V22A	V5, V16A, V26	739	511	410	35	Fr	24R	749	V27	829	590	729	M8B, M8BK	601	588 X26	546	418	748	419	413	X13	411	X1
Motility	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	+	−
Indol	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+	+
H_2S	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+
Cellobiose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	−	−
a-Methylglucoside	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	−	−	−	⊕	⊕	−	−	−	−	⊕	−	−	−	−	−	⊕	−
Dulcitol	−	−	−	⊕	⊕	−	⊕	−	⊕	−	⊕	−	−	⊕	⊕	−	−	⊕	⊕	⊕	⊕	−	−	⊕	−
Salicin	−	⊕	⊕	+D	⊕	⊕ S	−	−	+D	±D	+D	⊕ S	−	+D	+D	⊕	+D	⊕	⊕	⊕	±D	⊕	⊕	⊕	⊕
Sorbitol	⊕	⊕	−	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	?	⊕	⊕	⊕	⊕	⊕	−	⊕	⊕	⊕	⊕
Raffinose	+	⊕	⊕	+	⊕	+	+	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	⊕	−
Pectin	−	⊕	⊕	−	−	−	±	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Aesculin	−	⊕	⊕	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	⊕
Amygdalin	±	+	+	−	±	−	−	−	−	−	−	−	−	±	−	±	−	−	±	−	−	−	−	−	−
Litmus milk	+	+C	+C	+C	+C	+C	+	+	+	+C	+	+C	+C	+C	+	+C	+C	+C	+C	+C	+C	+C	+C	+C	+C

• *Note:* ⊕ = acid and gas; + = acid only, or positive test; ±-slight acidity; − = no fermentation, or negative test; S = slow fermentation, 4 to 7 days; D = delayed fermentation, 7 to 14 days; C = coagulated.

shown in table 3. With the exception of two cultures, 411 and Xl, they fermented cellobiose with the production of acid and gas. All, except four, were motile by both the hanging drop and soft agar methods. Approximately two-thirds utilized the citrate radical and produced hydrogen sulphide, but no uniform correlation between these metabolic activities was found. About one-half fermented *a*-methylglucoside and produced indol, but again no correlation was observed. Thus, it was evident that most of these cultures differed from one another in one or more respects and that the group was heterogeneous. Indeed, on the basis of each of these differences, the 29 cultures yielded no less than 25 different combinations of characteristics.

TABLE 4

Differential characteristics of major groups

	NUMBER OF CULTURES					
	9	4	8	6	1	1
Cellobiose	⊕	⊕	⊕	⊕	−	−
Citrate	+	+	+	−	+	−
H_2S	+	−	+	−	−	+
a-Methylglucoside	⊕	⊕	−	−	−	−

Symbols as in table 3.

On the basis, however, of the characteristics which served to designate these cultures as Escherichia-Aerobacter "intermediates," namely the utilization of citrate, the fermentation of cellobiose and *a*-methylglucoside and the production of hydrogen sulphide, they could be divided into 6 major groups, as indicated in table 4.

In table 5 are set forth the differential characteristics of the members of the cellobiose, *a*-methylglucoside and citrate positive group. Strains V5, V16A and V26 are identical, but otherwise no two cultures gave exactly the same combination of characteristics.

Members of the cellobiose, citrate and hydrogen sulphide positive, and *a*-methylglucoside negative group (table 6) did not differ among themselves as much as those in the previous group.

Thus, there was uniform correlation between certain characteristics, i.e., all were motile, failed to produce indol or to ferment aesculin, inositol and pectin, and all fermented sorbitol.

TABLE 5

Differential characteristics of the cellobiose, a-methylglucoside and citrate positive group

CULTURE	691	V22A	V5, V16A, V26	739	511	410	55	Fr	24R	749	V27
Motility	+	+	+	+	+	+	+	+	+	+	−
H_2S	+	−	−	+	+	+	+	+	+	+	+
Indol	+	+	+	−	−	−	−	−	−	−	−
Sucrose	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	−	−	−
Dulcitol	−	−	−	⊕	⊕	−	⊕	−	⊕	−	⊕
Salicin	−	⊕	⊕	+D	⊕	⊕S	−	−	+D	±D	+D
Raffinose	+	⊕	⊕	+	⊕	+	+	−	−	−	−
Sorbitol	⊕	⊕	−	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Inositol	−	−	−	−	−	−	−	+	−	−	−
Pectin	−	⊕	⊕	−	−	−	±	±	±	−	−
Aesculin	−	⊕	⊕	−	−	−	−	−	−	−	−
Amygdalin	±	+	+	−	±	−	−	−	−	−	−

Symbols as in table 3.

TABLE 6

Differential characteristics of the cellobiose, citrate and H_2S positive, and a-methylglucoside negative group

CULTURE	529	590	729	M8B, M8BK	601	588, X26
Motility	+	+	+	+	+	+
Indol	−	−	−	−	−	−
Sucrose	⊕	⊕	−	−	−	−
Dulcitol	−	−	⊕	⊕	−	−
Salicin	⊕S	−	+D	+D	⊕	+D
Raffinose	+	+	−	−	−	−
Amygdalin	−	−	±	−	±	−
Aesculin	−	−	−	−	−	−

Symbols as in table 3.

No two cultures of the cellobiose positive, *a*-methylglucoside, citrate and hydrogen sulphide negative group gave the same combination of reactions (table 7). The only correlation found

was between sucrose and raffinose fermentation. All but one produced indol, and only one fermented sucrose.

The differential characteristics of two cellobiose negative cultures, 411 and X1, are shown in table 3. These cultures possessed several characteristics which were exceptions to the entire group. Thus, 411 was the only culture to produce hydrogen sulphide which failed to utilize citrate and ferment cellobiose; X1 was the only one which utilized citrate and failed to ferment cellobiose. It was also the only culture which failed to ferment *d*-arabinose, *l*-xylose and gentiobiose (table 2).

TABLE 7

Differential characteristics of the cellobiose positive, a-methylglucoside, citrate and H_2S negative group

CULTURE	546	418	748	419	413	X13
Motility	+	+	+	+	−	−
Indol	+	+	+	+	+	−
Sucrose	⊕	−	−	−	−	−
Dulcitol	⊕	⊕	⊕	⊕	−	−
Salicin	⊕	⊕	⊕	±D	⊕	⊕
Sorbitol	⊕	⊕	⊕	−	⊕	⊕
Raffinose	+	−	−	−	−	−
Melibiose	⊕	−	⊕	⊕	⊕	⊕
Aesculin	−	−	±D	−	−	+
Adonitol	−	−	⊕	−	−	−
Pectin	−	−	−	−	−	−
Amygdalin	−	−	−	−	−	−

Symbols as in table 3.

No conclusions could be drawn from our many attempts to determine starch and dextrin fermentation because the results were irregular not only with a single method, but also when different methods were employed. Hence, the detailed data will not be presented. On some occasions all of the cultures produced acidity in starch broth, while at other times most of them failed to do so. Irregularities were also obtained on starch agar (0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 per cent starch), although most cultures gave a negative reaction with this medium.

A comparison of our results and those of Werkman and Gillen

(1932) with cultures 55, 24R, M8BK and Fr shows certain discrepancies, except for culture 55, which behaved the same. Cultures 24R and M8BK, in our hands, produced hydrogen sulphide and fermented salicin, while culture Fr failed to produce indol or ferment salicin, raffinose and aesculin. Werkman and Gillen reported other findings with respect to these characteristics.

DISCUSSION

The results of the detailed study of the cultural characteristics of 29 Escherichia-Aerobacter "intermediate" cultures show that they compose a heterogeneous group. It is impossible to reduce the number of different combinations of their cultural characteristics to less than 21. As pointed out above, and repeated here for the sake of emphasis, several media were used in some instances for the determination of a single characteristic. In each instance, with the single exception of starch fermentation, the different media elicited the same cultural reactions. Furthermore, during the course of the investigation, all of the cultures were tested repeatedly for many of their characteristics and the numerous sets of data obtained were identical. Finally, six months after the completion of the major investigation, all of the cultures were plated, and from 3 to 6 subcultures isolated from each parent culture. Many of the reactions were then redetermined for each sub-culture. Again, the results were in agreement. Hence, we believe that the differences between the cultures are intrinsic and cannot be attributed to mixed cultures, to inappropriate media or to errors in experimental technique.

For convenience, these cultures may be divided into 6 major groups on the basis of cellobiose and α-methylglucoside fermentation, citrate utilization and hydrogen sulphide production, as indicated in table 4. The selection of these characteristics is dictated partly by their intercorrelations and partly by the fact that they served to designate the cultures as Escherichia-Aerobacter "intermediates." It is important to note the absence of a reciprocal relationship between any two of these four characteristics.

At present, we do not wish to give generic allocation to any of these cultures, but rather to discuss their relationships to the

accepted Escherichia and Aerobacter genera (Bergey, 1934) and the proposed Citrobacter genus (Werkman and Gillen, 1932). It is possible to allocate all of them to the Escherichia genus. In fact, the sole use of Bergey's Classification (1934) offers no other choice. This system of classification does not include such characteristics as the methyl red reaction, the utilization of citrate and the fermentation of cellobiose, but common practice dictates their use. Furthermore, common opinion demands that typical Escherichia cultures should give a positive methyl-red reaction, fail to utilize citrate and not ferment cellobiose. Recently, Levine et al. (1932) reported that typical Escherichia and Aerobacter members do not produce hydrogen sulphide and their findings have been confirmed by us (unpublished data). Thus, it is evident that our cultures are similar to the Escherichia genus in that they give positive methyl-red tests and fail to produce acetyl-methyl-carbinol. On the basis of their ability to ferment cellobiose, utilize citrate and produce hydrogen sulphide, however, all of them differ from this genus in at least one characteristic, most of them in all three respects.

On the other hand, these cultures have characteristics in common with the Aerobacter genus, even though they may differ in some features. On the basis of their ability to utilize citrate and ferment cellobiose, all, except culture 411, are similar to members of this genus in at least one respect. They do not belong to the Aerobacter genus because they fail to produce acetyl-methyl-carbinol and are methyl-red positive. Thus, it is evident that our cultures are closely related to members of both the Escherichia and Aerobacter genera. Most of them are also similar to members of the proposed Citrobacter genus in that they fail to produce acetyl-methyl-carbinol, give positive methyl-red reactions and utilize citrate. The strains which utilized citrate and failed to produce hydrogen sulphide are different from those studied by Levine et al. (1932) who reported that all of their Voges-Proskauer negative and methyl-red positive cultures, which utilized citrate, produced hydrogen sulphide. A number of our cultures compose a slightly different group. They ferment cellobiose, but fail to utilize citrate. Similar cultures have been isolated from soil by Koser (1926); from human feces by Skinner and Brudnoy (1932),

and from water by Poe (1934). These strains are similar to typical members of the Escherichia genus. But, and here we agree with Koser, they should be considered as Escherichia-Aerobacter "intermediates" because of their ability to ferment cellobiose. Furthermore, we believe that they should not be considered in the same category as the citrate and hydrogen sulphide positive groups.

If the proposed Citrobacter genus is accepted on the basis that it "will be diagnosed with the purpose in mind of including the coli-aerogenes intermediates utilizing the citrate radical, methyl-red positive or weakly so and not producing acetoin from glycerol or glucose or in traces only from the latter," most of our cultures can be included in it. In fact, it would include cultures which differ from typical Escherichia members only in that they utilize citrate i.e., culture X1. On the other hand, citrate negative, cellobiose positive cultures and citrate negative, hydrogen-sulphide positive strains would be excluded. If cultures like X1 are given generic rank, the argument can be raised that the cellobiose positive, citrate negative group and the hydrogen-sulphide positive, citrate negative cultures also deserve similar consideration. Certainly such a system cannot be advocated. It is obvious, therefore, that the heterogeneity of the Escherichia-Aerobacter "intermediates" renders it extremely difficult, if not impossible, to establish a new genus which will separate them from both the Escherichia and Aerobacter genera and yet in itself be sufficiently inclusive. With the possible exception of trimethylene glycol production, hydrogen sulphide production is the only single characteristic which will separate any of these cultures from both the Escherichia and Aerobacter genera. It is not, however, sufficiently inclusive. If it can be shown that all of the Escherichia-Aerobacter "intermediate" types produce trimethylene glycol, while none of the typical members of the Escherichia-Aerobacter genera do so, we are willing to admit that the acceptance of the proposed Citrobacter genus may be justifiable. A simple and rapid test for the detection of trimethylene glycol is essential if this characteristic is to have practical significance in bacterial taxonomy.

It would seem to us that the Gram-negative, non-sporogenous, aerobic bacilli which produce acid and gas from lactose can be divided into the Escherichia and Aerobacter genera on the basis of acetyl-methyl-carbinol production. Thus, the Escherichia-Aerobacter "intermediates" would be allocated to the Escherichia genus. Such characteristics as the utilization of citrate, the production of hydrogen sulphide and the fermentation of cellobiose and *a*-methylglucoside could be employed as the means for distinguishing species or types within the genus. A classification of this type groups these closely related cultures in a single genus, and yet recognizes well-defined species differentiations.

SUMMARY

A detailed study of the cultural characteristics of 29 methyl-red positive and Voges-Proskauer negative Escherichia-Aerobacter "intermediate" cultures is presented. On the basis of citrate utilization, hydrogen-sulphide production and the fermentation of cellobiose and *a*-methylglucoside, these cultures can be divided into 6 major groups, but the existence of 25 sub-groups is brought out by additional tests for motility, the production of indol and the fermentation of other carbohydrates. These facts indicate the heterogeneity of the group. No single characteristic has been found which will differentiate all the members of this "intermediate" group from both the Escherichia and Aerobacter genera. The relationship of these cultures to the Escherichia and Aerobacter genera is discussed, and the opinion is expressed that they should be allocated to the Escherichia genus.

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THE FERMENTATION OF ALPHA-METHYLGLUCOSIDE BY BACTERIA[1]

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Koser and Saunders (1932) found that *a*-methylglucoside was not fermented by typical cultures of *Bacterium coli*, while members of the Aerogenes group usually attacked it with the production of acid and gas. They reported positive fermentation also by the majority of the methyl-red positive, Voges-Proskauer negative, citrate and cellobiose positive "coli-aerogenes intermediate" types isolated from soil. Later (1933), these workers showed that comparatively few bacteria ferment this carbohydrate; in particular, of the 24 species studied, *Bacterium aerogenes*, *Bacterium Friedländeri* and *Proteus vulgaris* were the only active fermenters, while streptococci from scarlet fever, septic sore throat and erysipelas and the pneumococci decomposed it slowly. These findings suggested that the fermentation of *a*-methylglucoside might serve as a convenient additional taxonomic characteristic, particularly in the case of the Escherichia-Aerobacter group. Accordingly, the ability to ferment *a*-methylglucoside has been determined for a large number of bacterial species, belonging in many genera. The results are presented in this report.

METHODS AND MATERIALS

A total of 730 cultures, representing approximately 60 species or groups, was employed. This number includes 472 Escherichia-Aerobacter strains isolated from human urine and feces and from

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water. The rest were selected from the laboratory collection. The characteristics of most of the cultures were determined in detail to permit exact taxonomic allocation. An exception was made, however, for the sporogenous cultures, in which case the species designations listed are those given by the laboratories from which the cultures were obtained.

The test medium was prepared by adding *a*-methylglucoside (Eastman), to yield a concentration of 0.5 per cent, to nutrient broth, composed of 0.3 per cent meat extract (Bacto) and 0.5 per cent peptone (Bacto), which had been adjusted to pH 7.2. Bromcresol purple served as an indicator. The broth was dispensed in the Durham type of fermentation tube, and sterilized by autoclaving for twenty minutes at 15 pounds pressure.

Incubation was carried out at 37°C. Observations were made for two weeks, unless both acid and gas were produced sooner. The results were recorded daily during the first week, and every second or third day during the second.

RESULTS

The results of the tests for *a*-methylglucoside fermentation by all of the genera and species studied are recorded in table 1. Relatively few were able to utilize this carbohydrate although all, with the exception of *Alcaligenes faecalis*, fermented glucose. All of the Aerobacter cultures, regardless of species, decomposed the glucoside with the production of acid and gas, but none of the typical members of the Escherichia group were able to do so. Many of the Escherichia-Aerobacter "intermediate" strains produced acid and gas, but the majority of them failed to utilize the carbohydrate. The Proteus cultures differed significantly in their ability to attack the glucoside. Proteus X19 and X2, and one unclassified strain produced acid only, while *Proteus mirabilis*, Proteus XK and 16 unclassified strains failed to show evidence of fermentation. *Bacillus subtilis* (Ford) was the only culture of the spore-forming group which fermented *a*-methylglucoside. Because of this exceptional behavior, it was replated several times and subcultures isolated. These sub-cultures behaved exactly like the parent culture. The behavior of *Gaffkya tetra-*

TABLE 1

Alpha-methylglucoside fermentation

ORGANISMS	NUMBER OF STRAINS	FERMENTATION	ORGANISMS	NUMBER OF STRAINS	FERMENTATION
Aerobacter aerogenes	34	AG	Shigella paradysenteriae (Sonne)	19	
Aerobacter oxytocum	27	AG	Shigella dispar	11	
Aerobacter cloacae	16	AG	Shigella alkalescens	2	
Aerobacter levans	2	AG	Shigella ambigua	2	
Aerobacter unclassified	21	AG	Pseudomonas "pyocyaneus"	4	
Escherichia-Aerobacter "intermediates"	20	AG	Flavobacterium vitarumen	1	
Escherichia-Aerobacter "intermediates"	49		Flavobacterium unclassified	1	A
Escherichia "coli" group	208		Chromobacterium violaceum	1	
Escherichia "communior" group	95		Serratia unclassified	7	
Alcaligenes faecalis	3		Acetobacter unclassified	1	
Proteus mirabilis	2		Bacillus cereus	1	
Proteus X19 (Felix)	2	A	Bacillus megatherium	1	
Proteus X19	2	A	Bacillus mycoides	1	
Proteus XK (Kingsbury)	2		Bacillus niger	1	
Proteus X2 (Felix)	2	A	Bacillus subtilis (Koch)	1	
Proteus unclassified	16		Bacillus subtilis (Marburg)	1	
Proteus unclassified	1	A	Bacillus subtilis (Soule)	1	
Salmonella suipestifer	3		Bacillus subtilis (Ford)	1	A
Salmonella aertrycke	4		Bacillus pseudotetani	1	
Salmonella enteriditis	19		Bacillus anthracis	1	
Salmonella Schottmülleri	8		Bacillus unclassified	5	
Salmonella paratyphi	8		Staphylococcus aureus	13	
Salmonella gallinarum	18		Staphylococcus albus	4	
Salmonella pullorum	36		Neisseria catarrhalis	2	
Salmonella Morgani	4		Sarcina citrea	1	
Hirshfeld's bacillus	1		Sarcina ureae	1	
Voldagsen's bacillus	1		Rhodococcus rhodochrous	2	
Eberthella typhi	13		Gaffkya tetragena	1	A*
Shigella dysenteriae (Shiga)	3		Micrococcus aurantiacus	1	A*
Shigella paradysenteriae (Flexner)	8		Micrococci unclassified	12	
Shigella paradysenteriae (Hiss and Russell)	1		Total number of strains	730	

AG = acid and gas; A = acid only.

* Delayed fermentation.

gena and *Micrococcus aurantiacus* was similar to that of certain streptococci studied by Koser and Saunders (1933). A definite acidity was not produced until the second week of incubation.

The relation of *a*-methylglucoside fermentation to the common taxonomic characteristics of the Escherichia-Aerobacter cultures, and to their sources, is shown in table 2. The Aerobacter and "typical" Escherichia strains showed perfect correlation with all of these differential tests. In the Escherichia-Aerobacter "intermediate" group, however, the only reciprocal relationship found was that between the methyl-red and the Voges-Proskauer reactions. Of the 69 cultures tested, 61 fermented cellobiose; 43 utilized citrate and 20 fermented *a*-methylglucoside. Thus, a better correlation was shown between the fermentation of *a*-methylglucoside and the Voges-Proskauer reaction, than between either the fermentation of cellobiose or the utilization of citrate and the Voges-Proskauer reaction. No connection was found between *a*-methylglucoside fermentation and the source of the cultures; in fact, no reciprocal relationship was observed

TABLE 2

Correlation of alpha-methylglucoside fermentation with other differential tests of the Escherichia-Aerobacter cultures and with their sources

GROUP	SOURCE	NUMBER OF CULTURES	METHYL RED TEST		VOGES-PROSKAUER TEST		UTILIZATION OF CITRATE		FERMENTATION OF CELLOBIOSE		FERMENTATION OF *a*-METHYL-GLUCOSIDE	
			+	−	+	−	+	−	+	−	+	−
Aerobacter	Urine	27	0	27	27	0	27	0	27	0	27	0
	Feces	20	0	20	20	0	20	0	20	0	20	0
	Water	53	0	53	53	0	53	0	53	0	53	0
Escherichia	Urine	190	190	0	0	190	0	190	0	190	0	190
	Feces	90	90	0	0	90	0	90	0	90	0	90
	Water	23	23	0	0	23	0	23	0	23	0	23
Escherichia-Aerobacter "intermediates"	Urine	24	24	0	0	24	9	15	19	5	0	24
	Feces	16	16	0	0	16	12	4	15	1	7	9
	Water	20	20	0	0	20	13	7	18	2	6	14
	Soil	9	9	0	0	9	9	0	9	0	7	2
Total		472	372	100	100	372	143	329	161	311	120	352

between any of the differential characteristics and the source of the bacteria.

DISCUSSION

It is evident that the substitution of a methyl group for the hydrogen of the hydroxyl group attached to the number one carbon atom of glucose markedly influences the availability of the carbohydrate for bacteria, as reported by Kendall and Gross (1930) and by Koser and Saunders (1933).

The results obtained with the Escherichia-Aerobacter group agree with those of Koser and Saunders (1932) and Poe (1934). They are also in accord with the findings of Kendall and Gross (1930) for Escherichia cultures and for *Aerobacter cloacae*, but not for *Aerobacter aerogenes*. These workers, using a single strain, reported that "*B. lactis-aerogenes*" failed to ferment *a*-methylglucoside. It is evident from the observations of Hees and Tropp (1926), Koser and Saunders (1932) and ourselves, however, that *Aerob. aerogenes* ferments *a*-methylglucoside.

In the cases of the Aerobacter and typical Escherichia strains, the fermentation of *a*-methylglucoside correlates perfectly with the production of acetyl-methyl-carbinol, the methyl-red reaction, the utilization of citrate and the fermentation of cellobiose (table 2). For the separation of these two groups, therefore, the fermentation of *a*-methylglucoside can be employed as well as any other of the reactions. On the other hand, this characteristic cannot be used alone to differentiate the Escherichia-Aerobacter "intermediate" cultures from both Aerobacter and typical Escherichia strains. It may be usefully employed, nevertheless, as a means of distinguishing species or types within this "intermediate" group. It is significant that in the utilization of *a*-methylglucoside most of the "intermediate" strains are similar to typical Escherichia.

The findings show that neither the fermentation of *a*-methylglucoside nor any other characteristic, alone or in combination, will indicate whether or not the source of Escherichia-Aerobacter strains is fecal or non-fecal. Furthermore, they emphasize the fact that Aerobacter and Escherichia-Aerobacter "intermediate" cultures may be found in human excreta.

The results obtained with the Proteus cultures (table 1) indicate that the fermentation of *a*-methylglucoside may be used advantageously for distinguishing certain closely related species or types. Our cultures of *Proteus mirabilis* failed to attack the glucoside, while Kendall and Gross and also Koser and Saunders (1933) reported that *Proteus vulgaris* fermented it. Proteus XK also failed to ferment *a*-methylglucoside, while the closely related Proteus X strains decomposed it.

SUMMARY

1. The ability to ferment *a*-methylglucoside has been determined for 730 bacterial cultures representing 19 genera and more than 60 species.

2. Few species were able to attack the glucoside.

3. All of the Aerobacter cultures and approximately one-third of the Escherichia-Aerobacter "intermediate" strains produced acid and gas, while none of the "typical" Escherichia showed evidence of any fermentative power.

4. The relation of *a*-methylglucoside fermentation to other differential characteristics of the Escherichia-Aerobacter group, and to their sources, is recorded.

5. The fermentation of *a*-methylglucoside will differentiate Aerobacter from "typical" Escherichia strains and can be employed advantageously for distinguishing species or types within the Escherichia-Aerobacter "intermediate" group. On the contrary, it will not differentiate "intermediate" cultures from both Aerobacter and "typical" Escherichia strains or indicate whether or not the source of Escherichia-Aerobacter cultures is fecal or non-fecal.

6. The value of *a*-methylglucoside fermentation as an additional aid in the separation of certain closely related Proteus cultures is indicated.

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THE RELATIONSHIP OF SOIL PROTOZOA TO TUBERCLE BACILLI[1, 2]

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The aim of the work outlined in this paper was to determine what rôle protozoa play in the destruction of tubercle bacilli in nature and itwas carried out simultaneously with studies on the death rate of tubercle bacilli in soil and in association with various bacteria and fungi.

ISOLATING SOIL CILIATES

Preliminary studies on the problem dealing with the relation of protozoa to acid-fast bacteria, especially the tubercle bacilli, were largely concentrated on the relationship of a certain ciliate to several pure cultures of bacteria. This protozoan was found to be present in great abundance in a soil taken from a plot of the New Jersey Agricultural College and Experiment Station. The organism was found to develop readily on many bacteriological media tested. It has been identified as *Colpoda Steinii*.

As the relationship could not be determined with certainty in mixed cultures similar to the mixed population in which protozoa naturally live in soil, a method of purifying the cultures was undertaken. The procedure of repeated picking and washing the ciliate was similar to that employed by Peters (1921). A single protozoan was picked with a sterile micropipette. This was accomplished readily under a dissecting microscope. The protozoan was placed in sterile medium in a depression slide. After

[1] Journal Series Paper, New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

[2] This work was carried out under the auspices of the Committee on the Microbiology of the Soil of the National Research Council.

about five minutes the operation was repeated and thus continued through seven sterile droplets. The protozoan was finally placed in the desired pure culture of bacteria and allowed to multiply. Control protozoa inoculated into nutrient broth and tested for purity were found to be free from bacteria. In the above manner the protozoan was successfully introduced into several pure cultures of bacteria in which it thrived. One of the bacteria was an unidentified small Gram-positive bacillus found to be predominating in a mixed infusion in which the protozoan grew excellently. The protozoan was later transferred to a culture of *Bacillus megatherium*, in which it grew profusely. It failed to thrive at a temperature of about 28° to 31°C. in an *A. aerogenes* culture although on some occasions the introduced cells multiplied readily. When the temperature was lowered to about 23° to 25°C., the protozoan was found to thrive with *A. aerogenes*. During the summer months placing the test tubes in a pan of water in a draft of air caused a lowering of temperature favorable to this ciliate.

There is no reasonable doubt as to the purity of the bacterial cultures in which the protozoan was induced to grow. Control transfers of the protozoa through sterile media constantly proved them to be free from bacteria, because of the failure to develop bacterial growth in nutrient broth or on nutrient agar. The protozoa could thus be freed of all the pure bacterial cultures in which they were grown. These bacteria would have quickly produced turbidity in nutrient broth. As an additional precaution to assure purity, the protozoan was transferred twice from each pure culture to another pure culture of the same bacterium, using the identical procedure employed in the original purification of the cultures. Culture studies did not show the presence of contaminating bacteria.

Three cells of protozoa freed from bacteria by transferring with a pipette through a sterile media were placed in each of three nutrient broth suspensions of Timothy 1589, a saprophytic acid-fast organism. The suspensions were observed for a period of one week. One and two days after the transfer many protozoa

were found to be present. Multiplication of the protozoan had actually occurred. The numbers were, however, rapidly reduced and after the seventh day the protozoa were no longer observed and could not be revived by transferring to fresh suspensions. Similar experiments with Avian 531 suspensions revealed that multiplication also occurred but even less readily than with the Timothy 1589 suspensions and no protozoa remained after three or four days. Bacteriological studies proved that no contamination had occurred during the manipulation. These experiments were conducted at summer room temperature which was later found to be too high for the best growth of the protozoan. Better success was obtained with the growth of the ciliate in Timothy 1589 and Avian 531 suspensions later, when a more convenient method of transferring the protozoan had been devised.

Because of the very tedious task of obtaining a sterile protozoan by the pipetting method and because only one cell could be introduced into a bacterial culture at a time, a more satisfactory method was sought. The protozoan encysted upon drying and the cysts possessed considerable resistance to heat and chemicals. The active protozoan in a liquid culture was destroyed by a temperature of 60°C. in less than five minutes. A dried culture resisted 90°C. for about twenty minutes and 85°C. for over two hours. *Aerobacter aerogenes* was found to be rather easily destroyed after drying. The cultivation of the protozoan in a culture of *A. aerogenes* had already been accomplished. This pure culture of the protozoan and the bacterium was incubated at 23° to 25°C. One drop of a four-day culture swarming with protozoa was added to a series of sterile test tubes. The moisture was allowed to evaporate in an incubator kept at 37°C. The dry tubes were heated in an oven to determine the temperature and time necessary to destroy the bacteria without destroying the protozoan cysts.

The medium used was found to alter greatly the thermal death point of the dry *A. aerogenes*. The bacteria in the drop of medium designated *B* resisted 95°C. for one hour, while the bacteria in the dried drop from another medium, *A*, were almost always

destroyed by a temperature of 70°C. in one hour. The formulae for these media are:

"A" medium

Soil extract	100.0 cc.
Peptone	0.25 gm.
Water	900.0 cc.

"B" medium

Soil extract	400.0 cc.
Peptone	1.0 gm.
Water	600.0 cc.

Soil extract

Soil	1000.0 gm.
Water	1000.0 cc.

Boil ten minutes and filter

Bacteria become most numerous in the richer *B* medium. The difference in the thermal death point is probably influenced by the number of cells.

A dried drop of the four-day culture in the *A* medium heated at 70°C. for one hour proved to be usually bacteria free, while the protozoa remained viable. Cultures of the protozoan could be conveniently obtained by merely adding a pure culture of a bacterium to a test tube containing bacteria-free cysts. To determine the success of the method, pure cultures of the tiny Gram-positive bacillus were introduced into the test tubes containing the protozoan cysts. Active protozoa could be observed after one day. After several days, the protozoa swarmed through the media. Transfers from each tube to a d-glucose broth fermentation tube were then made. When *A. aerogenes* survived, gas formation was readily detected. The Gram-positive bacillus failed to produce gas in the d-glucose broth. Tests were developed for the detection of *A. aerogenes* in the presence of other bacteria and were used to check all the transfers. The organism, *A. aerogenes*, was not destroyed in about 15 per cent of the heated tubes.

When nutrient broth was introduced into a test tube containing the protozoan cysts, excystation was brought about. Active protozoa could be noted in one or two days. After about the

third day, no active protozoa were present. Bacteria-free broth failed to support growth of the protozoa.

Nutrient broth suspensions of Timothy 1589, Avian 531 and an acid-fast soil bacillus were placed in the test tubes containing the bacteria-free protozoan cysts. In the Timothy 1589 and Avian 531 cultures slow development of the ciliate occurred. The cultures were continued by making transfers to fresh suspensions of the bacteria. The ciliate failed to grow in suspensions of an acid-fast bacillus from soil; a few ciliates were still present on the fifth day; thereafter none were observed. No contamination of cultures occurred to detract from the accuracy of the observations. After two months cultivation, three cultures of the protozoan with the Avian 531 organism and three cultures of the protozoan with the Timothy 1589 organisms were still flourishing. Transfers to fresh suspensions were made weekly. Apparently the cultures could be maintained in this manner indefinitely. *Colpoda Steinii* has now been cultured with Avian 531 for more than one year. No foreign bacteria have entered the cultures. Transfers of the Avian 531 culture to nutrient broth did not become turbid nor did the protozoan live in this clear broth. If a contaminating organism capable of supporting the ciliate had been present, the ciliate would have continued to exist in the nutrient broth. No development of colonies occurred when the Avian 531 culture was plated on nutrient agar.

In later tests, *Colpoda Steinii* failed to develop in suspensions of *M. ranae*, a frog tubercle bacillus and in suspensions of eleven acid-fast bacteria of soil origin. One soil acid-fast bacillus furnished luxuriant growth of the protozoan. The H 37 human tubercle bacillus and the Ravenel bovine tubercle bacillus failed to support *C. Steinii*.

C. Steinii did not grow in the filtrate from a six weeks glycerol broth culture of Avian 531. The soluble constituents of the culture did not alone fulfill the food requirements of the protozoan.

CULTURAL RELATIONSHIPS OF ACID-FAST BACTERIA AND CILIATES

This group of experiments was devised for the purpose of determining the effect of protozoa upon acid-fast bacteria and

acid-fast bacteria upon protozoa. The main possibilities considered are:

1. The protozoa may be parasitized by the acid-fast bacteria and serve as carriers of disease, as many insects serve as carriers for mammalian parasites.

2. The acid-fast bacteria may be toxic to the protozoa and cause abnormalities in their functions and structures or retard normal development.

3. The protozoa may utilize the bacteria as food and ingest and destroy them.

A technic was devised for staining ciliates by the acid-fast method in order to determine whether ingestion of Mycobacteria occurred. The fixative found to be most successful was of the formula:

Picric acid	1.0 gm.
Alcohol	200.0 cc.
Formalin	40.0 cc.
Glacial acetic acid	15.0 cc.

Equal parts of fixative and culture are mixed and allowed to stand at 37°C. for thirty minutes. Excess fluid is removed by centrifugation and 0.05 per cent egg albumin added. The excess fluid is again removed and the material spread on a glass slide, allowed to dry slowly and stained by the Ziehl-Nielsen method.

Pure cultures consisting of *C. Steinii* and Avian 531 together and likewise *C. Steinii* with Timothy 1589 were thus stained. Many acid-fast bacteria could be seen within the protozoa. The number contained in some single protozoa was estimated at about 100. Large clumps of acid-fast material were dispersed throughout the ciliate and many intact bacilli were noted. Undoubtedly ingestion had occurred.

The problem was attacked upon the assumption that if multiplications of the acid-fast bacteria occurred within ciliates, the acid-fast bacteria would persist through repeated transfers of the protozoa to a fresh medium containing no acid-fast bacteria. Timothy 1589 is able to support the protozoa indefinitely when fresh transfers to nutrient broth are repeatedly made at intervals of about one week if surface growth of the bacterium occurs.

As the bacteria then develop outside of the protozoa this fails to prove anything. Avian 531 fails to grow readily at the temperature, about 23°C., favorable to the protozoa. A frog tubercle bacillus was therefore procured from the Henry Phipps Institute at Philadelphia and used in the experiments. Six mixed cultures consisting of known pure cultures were prepared. All contained a tiny Gram-positive soil bacillus and in addition:

1. *C. Steinii* and *Avian 531*
2. *C. Steinii* and *Timothy 1589*
3. *C. Steinii* and *M. ranae*
4. *C. cuculus* and *Avian 531*
5. *C. cuculus* and *Timothy 1589*
6. *C. cuculus* and *M. ranae*

The tiny Gram-positive bacillus served excellently to support growth of the protozoa. The medium with which the cultures were carried was such that the acid-fast bacteria were favored by the presence of ammonia, phosphate and glycerol. Nutrient broth furnished nutrients for sparse growth of the tiny Gram-positive bacillus which could not use the glycerol. All the mycobacteria were capable of growing on the surface when carefully planted, even with the Gram-positive bacteria contaminating the liquid.

Medium "D"

Nutrient broth	50.0 cc.
Water	950.0 cc.
Glycerol	10.0 cc.
Ammonium sulfate	1.0 gm.
Phosphate buffer	10.0 cc.

Phosphate Buffer

K_2HPO_4	60.0 gm.
KH_2PO_4	20.0 gm.
Water	1000.0 cc.

After twenty-four hours, stains revealed that acid-fast bacteria had been ingested by the protozoa of all cultures. The presence of acid-fast bacteria did not depress the numbers of protozoa nor cause observable damage or stimulation to them. The next day all cultures were transferred, 1 cc. of culture being

added to 10 cc. of sterile medium. After five days, examination revealed that some, but only a very few, acid-fast bacteria were contained in the protozoa. Fresh transfers were made as before. After five days examination was made again. No acid-fast bacteria could be found. This indicated that multiplication of acid-fast bacteria did not occur within ciliates, even when the medium in which the ciliates grew was very favorable for acid-fast strains. Jordan (1932) detected no evidence that tubercle bacilli multiplied within *Colpidium campylum*.

To determine the effect of ciliates on the acid-fast bacterial numbers, pure cultures of the following composition were prepared:

1. Gram-positive bacillus and *Avian 531*
2. Gram-positive bacillus *C. Steinii* and *Avian 531*
3. Gram-positive bacillus *C. Steinii* and *M. ranae*
4. Gram-positive bacillus *C. cuculus* and *Avian 531*
5. Gram-positive bacillus *C. cuculus* and *M. ranae*
6. Gram-positive bacillus and *C. cuculus*

Medium *D* was used because it favored acid-fast bacteria and because the protozoa developed profusely in it when the tiny Gram-positive bacillus was included. A sufficient population of protozoa could not be obtained when the tiny Gram-positive bacillus was omitted. Of the medium, 5 cc. amounts were inoculated with 1 cc. of desired culture without the acid-fast bacteria and allowed to incubate for three days at 23°C. To 5 cc. amounts of sterile medium, 1 cc. transfers were again made and allowed again to incubate for three days so that the protozoa would be numerous. One cubic centimeter of a *D* medium, acid-fast suspension was added to each culture as desired; the mycobacteria had been dispersed by shaking with glass beads.

Twenty-four hours later all bacteria were counted by a special technic. An aliquot was taken and the Gram-positive bacilli counted by ordinary plating. Then to each suspension was added 5 cc. of 2 per cent sodium hydroxide. Five minutes action was permitted and the material was plated on crystal-volet-glycerol-agar. On these plates only acid-fast bacteria developed. *M. ranae* failed to form colonies so no counts were obtained for it.

Each culture was duplicated. The counts are:

Group	Protozoa	Gram-positive bacilli	Avian 531
1. (No protozoa)	0/cc.	520,000,000/cc.	84,000/cc.
2. (With *C. Steinii*)	2,000	20,000,000	40,400
4. (With *C. cuculus*)	1,000	39,000,000	9,200

The above technic was repeated to check results. Counts were made 12 hours and 60 hours after the mycobacteria were added. The Avian 531 counts are given below.

Time after Avian 531 was added	Culture with *C. Steinii*	Culture without *C. Steinii*
12 hours	162,000	660,000
60 hours	1,640,000	1,600,000

An additional experiment conducted after experimental methods of counting Avian 531 were more satisfactorily worked out revealed that Avian 531 numbers changed from 15,000,000 to 25,000,000 per cubic centimeter in *C. Steinii* and in *C. cuculus* cultures in two weeks. The plate count was the same for the culture without protozoa. The same Gram-positive bacillus used in the previous experiments was included. Each count was based on the average of 6 plates, and each count was duplicated. It was thus demonstrated with certainty that the protozoa did not have a pronounced destructive action on this tubercle bacillus. The slight observed increase in Avian 531 is not of significant magnitude to indicate an actual increase in Avian 531 numbers. There is generally an increase in the plate count of Avian 531 following dispersion in a liquid medium, conditions being similar to those of this experiment.

There was considerable evidence that an excessive concentration of tubercle bacilli in a medium was unfavorable to protozoa. When a very concentrated Avian 531 suspension was added to a culture of protozoa in a bacterial medium well suited for rapid development of the protozoa, the protozoa were decidedly inhibited as evidenced by population. Thus, Avian 531 added in excessive quantities to vigorous *C. Steinii* and *C. cuculus* cultures on *A. aerogenes* or other bacteria diminished the multiplication rate of these protozoa. This is easily explained, for Avian 531 is a poor food for *C. Steinii* as compared to many other bacteria.

When the protozoa are induced to ingest vast quantities of the poor food, the growth rate is diminished. Also, even a high concentration of bacteria better suited as protozoa food may check growth.

MORPHOLOGIC STUDIES OF THE ACID-FAST BACTERIA DURING INGESTION BY CILIATES

With due consideration for the prevalent knowledge of cellular digestion, it was deemed likely that marked changes in the morphology of the acid-fast bacteria would occur as a result of ingestion of ciliates. The ingested bacilli could be readily studied when stained by the acid-fast procedure. An extended study of the bacilli was therefore made.

The bacilli occurring within *C. Steinii* grown upon pure cultures of Timothy 1589 and Avian 531 were first observed. Large masses of acid-fast bacteria were found in which the individual organisms were often indistinguishable. This did not necessarily indicate a homogenization of the bacilli to a mass of acid-fast material, however, because whenever fewer bacilli occurred in a ciliate, the bacilli appeared to be unchanged from those of the original culture. The masses probably tended to appear homogenous because of the compact packing of the individual bacilli and because the decolorizer failed to remove the dye completely from the central portion of the ciliates. The tendency of dye retention was frequently noted.

Many observations of acid-fast bacteria were made at various time-intervals following the addition of mycobacteria to an active pure mixed culture of a ciliate and the tiny soil bacillus. The amount of mycobacteria suspension was so regulated that large masses did not accumulate, the individual bacilli thus being distinguishable within the protozoa. Some ingestion occurred immediately after addition of the acid-fast bacteria. During a week of observations of Avian 531 cultured with *C. cuculus* and *C. Steinii* no morphologic changes were noted as a result of ingestion. Observations were rendered somewhat difficult by the pleomorphism of Avian 531. The bacilli varied in size and shape and in degree of granulation. No morphological change has been attributed to ingestion of mycobacteria by *C. cuculus* or *C. Steinii*.

Isolation of pure cultures of a soil amoeba

Soil amoebae were isolated for the purpose of studying the relation of this group of organisms to the acid-fast bacteria. The isolation procedure is essentially the same as described by Severtzoff (1921).

A particle of soil was placed on the center of a cross produced on an agar plate by streaking a pure culture of *Aerobacter aerogenes*. The agar medium consisted of 100 cc. of nutrient broth, 900 cc. of water and 15 grams of agar. Less agar was used by Severtzoff, but the amount of exuded water caused the undesirable bacteria to spread too rapidly to the ends of the crosses. The plates were kept under bell jars at about 25°C. An amoeba was soon noted on the plates. After about a week transfers were made from the outermost portions of the crosses to the center of other similar plates. Transfers were repeatedly made in this manner. Microscopic examination of the plates revealed the progress of the amoeba along the streaks. Transfers were always made as soon as they reached the tip of the cross. A single amoeba was picked on one occasion so that the strain would be pure. After seven or eight transfers the amoeba was found to be in a pure culture of *A. aerogenes* as far as bacteriological studies could be used to determine the purity. Stained smears and colonies were typical of the pure culture. None of the bacteria survived toluol treatment for more than fifteen minutes.

It was necessary to free amoebae from the *A. aerogenes* in order that the amoebae could be introduced into pure cultures of acid-fast bacteria. Toluol treatment of the mixed culture was tried. The amoeba survived only for six minutes so this method could not be considered successful. Although other means could have been tried it was deemed best to seek a more resistant type of soil amoebae. Normal soil was treated with toluol for three hours and used for the purpose of isolation. It was believed that only protozoa which form very resistant cysts would survive. Many amoebae survived and two distinct types were induced to live upon pure cultures of *A. aerogenes*. Thirty minutes treatment with toluol destroyed the *A. aerogenes* and left the amoeba cysts viable. These cysts could then be transferred, free from

all contaminants, to any bacteriological medium or culture desired.

Amoebae and mycobacteria

Amoebae and acid-fast bacteria were introduced on the same agar plates to study the effect of the acid-fast bacteria upon the protozoa. All the culturing was done on an agar medium containing 100 cc. of nutrient broth and 15 grams of agar per liter. The amoebae were carried with pure cultures of *A. aerogenes*. The amoebae were added to all plates in aqueous suspensions taken from slant cultures. Acid-fast bacteria were added by means of a finely dispersed, filtered, aqueous suspension. Two distinctly different types of soil amoebae and three mycobacteria, Avian 531, *M. ranae*, and Timothy 1589 were used.

At first the acid-fast bacteria were added to the plates at the time the amoeba *A. aerogenes* suspensions were added. It was noted that amoebae were much retarded by the mycobacteria. No desirable active cultures were procured. Amoebae and acid-fast bacteria were successfully grown together when the amoebae were permitted to develop with *A. aerogenes* on an agar plate and the mycobacteria added after this development had occurred.

Preparations for staining were then made. The amoebae were suspended in 5 per cent acetic acid containing 0.1 per cent egg albumin by allowing the solution to wash about the surface of the plate. This material was placed on a glass slide and permitted to dry slowly. The Ziehl-Nielsen technic of staining was used. The conclusions drawn from the observations are:

1. Mycobacteria suspensions added to plates of agar medium hindered the development of amoebae.

2. Acid-fast bacteria placed on agar plates with amoeba soon collect about or within the amoeba cells.

3. Encystment is hastened by the mycobacteria.

4. No fat globules were noted in the amoebae with or without acid-fast bacteria, Sudan III being used as the fat stain; therefore, no abnormal fat distribution was observed.

5. The mycobacteria may have hindered the amoebae in several ways. (*a*) By toxic products. (*b*) By acting as indigestible

bodies which prevent normal feeding. (*c*) By a combination of factors.

No very good evidence can be here presented for any of these views. The acid-fast bacteria were not observably disintegrated by the amoebae. Therefore toxic products are somewhat difficult to account for. The great number of mycobacteria grouped about some amoebae indicated that these amoebae might have collected sufficent indigestible material to hamper normal digestion processes.

SUMMARY

1. Several methods were devised for conveniently and rapidly introducing pure cultures of protozoa, both ciliates and amoebae, into any pure culture of bacteria desired.

2. A culture of *Colpoda Steinii* was introduced into a nutrient broth suspension of Avian 531. The ciliate multiplied and was cultured thus for more than one year. Nutrient broth alone or the filtrate of a glycerol broth culture of Avian 531 did not support growth of the ciliate.

3. *Colpoda Steinii* and *Colpoda cuculus* do not destroy Avian 531, as evidenced by plate count studies.

4. Some acid-fast bacteria support growth of *C. Steinii*, others do not, a high degree of specificity being manifested.

5. Stains revealed large numbers of acid-fast bacteria to be ingested by *C. Steinii* and *C. cuculus*.

6. Several strains of soil amoebae failed to grow upon pure cultures of acid-fast bacteria.

7. Excessive concentrations of acid-fast bacteria inhibit protozoa.

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STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

IV. QUANTITATIVE ESTIMATION OF BACTERIAL GROWTH

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In carrying forward experiments on nutritional requirements of certain pathogenic bacteria, notably the diphtheria bacillus, the necessity for some fairly accurate method for determining the amount of growth became increasingly evident to the writer. It seemed wise to attempt a direct estimation, either of total dry weight of bacterial substance formed in a given amount of medium, or to estimate quantitatively some fairly constant constituent of the bacterial cell. The dry weight method, which has been used to some extent for other purposes by some workers, was ruled out because of the technical difficulty of filtration and washing of considerable numbers of cultures through bacteria-tight filters of a form suitable to be dried and weighed accurately. Nitrogen is a fairly uniform component of the living cell, and was selected as the simplest substance capable of rapid and careful determination. It has been found entirely practicable to centrifuge, wash and carry out micro-kjeldahl determinations on cultures of diphtheria bacilli. The method is somewhat tedious when many daily cultures must be dealt with, but the results obtained and information acquired have so far exceeded those possible with less quantitative records that we venture to hope others working in the same field may find the method of value. There is also every reason to believe that vaccines could be standardized by the same method. The difficulty of obtaining uniform dosage over periods of time by any of the usual methods

of counting or turbidity measurements, is well known, while dosage expressed in terms of bacterial nitrogen should be both easy to determine and constant in value. The attempt is being made to coördinate such values with those now employed in standardizing vaccines at the Massachusetts State Antitoxin Laboratory through the courtesy of Dr. Elliott S. A. Robinson.

Cultures are grown in fluid medium. Exactly 10 cc. of each lot to be tested are pipetted into a pyrex test tube 150 × 20 mm. in size with the end drawn to a blunt point like a centrifuge tube such as Pyrex no. 2185. The open end is without rim, but may conveniently be supplied with a small "pour out." The medium, after sterilization, is inoculated and incubated in a slanted position, in the case of the diphtheria bacillus, in order to allow maximum pellicle formation. In the accompanying chart, it will be seen that determinations carried out on duplicate tubes, during the early period of incubation, show rather wide differences in bacterial nitrogen, but that as maximum growth is attained, checks between duplicate tubes become fairly good, and in many cases, almost incredibly close. Later, there is a slow diminution of nitrogen, depending probably on ageing, death and partial autolysis of the cells. However, on the chart shown, at a temperature of 35°, the values are practically constant between forty-four hours and sixty-four hours. Routinely, therefore, we have grown our cultures for approximately fifty-four hours. Obviously, such a time curve would have to be made for any other type of organism which might show both more rapid growth and more pronounced autolysis.

The cultures, after removal from the incubator, are shaken and each tube is tilted repeatedly to wash down as much of the growth adhering to the wall as possible. They are then either autoclaved at 10 pounds for ten minutes, or steamed for fifteen minutes, the cotton plugs removed, and the tubes centrifuged in 100-cc. cups (International Equipment Co., no. 340), the space around the tube being about two-thirds filled with water to minimize risk of breakage. Five minutes' centrifugation at 2600 R.P.M. proves adequate for the diphtheria bacillus, although a longer time may be required for other organisms. The supernatant fluid is care-

fully pipetted off, and the sediment washed with 0.05 per cent acetic acid. If distilled water is used in the washing, a suspension is formed which does not readily come down in the centrifuge. The washing is done by stirring the sediment with a few drops of the dilute acetic acid, using a glass rod, which is then carefully rinsed with 2 cc. more of the acetic solution, and enough of the latter run in to fill the tube about two-thirds full, altogether about 20 cc., so that any pellicle adhering at the upper end of the original

TABLE 1

TIME OF INCUBATION	NITROGEN IN GROWTH ON 10.0 CC. MEDIUM	TIME OF INCUBATION	NITROGEN IN GROWTH ON 10.0 CC. MEDIUM
hours	mgm.	hours	mgm.
16	0.36 0.37	44	1.95 2.00
20	Lost Lost	48	1.98 1.99
24	0.84 1.03	52	2.00 2.04
28	1.36 1.52	56	2.00 2.00
32	1.62 1.82	60	2.00 2.02
36	1.97 1.95	64	2.01 2.02
40	2.05 1.91	72	1.94 1.97

slanted culture is covered. A second five minutes centrifugation is carried out, and the wash fluid drawn off. Calculation and determination of nitrogen in this wash water show that not enough remains in the tip of the tube to necessitate a second washing.

For the actual determination of the nitrogen, the Pregl (1930) micro-kjeldahl method has been used. Any other type of micro-kjeldahl would prove equally satisfactory, including colorimetric

methods, for the accuracy of the Pregl method is beyond the requirements of the rest of the procedure. However, since the

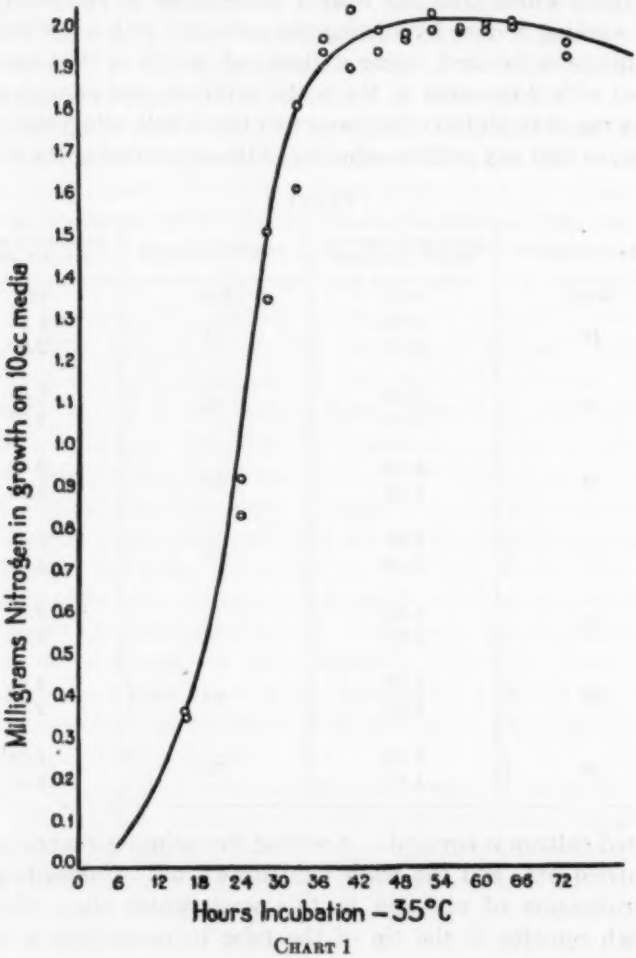

CHART 1

apparatus for this method was already assembled, it has been used and is probably as rapid as any other. For details of the method, the original description should be consulted. The digestion is

carried out directly in the culture tubes, and there is no other variation whatever from Pregl's procedure, except that we find it advantageous to make four additions of three drops each of 30 per cent H_2O_2 during the course of the digestion. The quantities of nitrogen dealt with in the studies made lie between about 0.2 and 2.0 mgm., or somewhat more, calling for from 5 to 15 cc., and very occasionally 20 cc. of 0.01 N HCl. It is possible to carry through determinations on about 20 cultures in a day.

The chart and table 1 show the type of result which may be expected.

SUMMARY

A method for the estimation of total quantity of bacterial growth by means of the micro-kjeldahl method is described. Its utility as a means of recording growth in studies on bacterial culture requirements is pointed out, and the suggestion is made that it would be found useful in vaccine standardization.

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FURTHER OBSERVATIONS ON CERTAIN VARIANTS OF BACILLUS MEGATHERIUM

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In a previous paper dealing with the variation of *Bacillus megatherium* (Knaysi, 1933), the author said:

> Most of these variants are non-sporogenic and it seems that spore formation is one of the characters most readily lost by *Bacillus megatherium*. Moreover, when the power to form spores is lost, there seems to be no tendency to regain it. Various strains thus changed have now been investigated for over a year for spore formation, but none has been seen yet. Colonies six and seven months old and still viable were free from spores.

Since this was written, the author had to be away for several months. During that time cultures of the various variants were kept in a cold room, the temperature of which fluctuates only a few degrees around the freezing point. Inoculations into meat infusion slants of pH 7.0 were made, the transfers were allowed to grow for twenty-four hours at room temperature and were then placed in the cold room. Eleven months later, the cultures were plated on meat infusion agar of pH 7.0 and the following observations were made:

All the cultures were viable with the exception of the budding strain. The forms inoculated had dissociated in some degree to other forms, but the original forms were still abundantly present and were readily recovered.

On examining for spores, it was found that the mucoid, the midget and the yellow variants formed spores readily and profusely. The colonies were identical in appearance to the non-

sporulating ones previously described and from which the present ones originated. The cells of the sporulating strains, however, have assumed, cytologically, certain characteristics of ordinary megatherium cells, for instance, the presence of the special granules lining the cell before spore formation takes place. The size and shape of the cells have also become more like the ordinary cells of the organism.

It is not certain whether the mucoid cells themselves have regained spore formation, or whether the midget forms, constantly present in abundance, are the ones responsible for the presence of spores in mucoid colonies.

This is, as far as the author knows, the first case of a spore-forming organism regaining spore formation after having lost it. The asporogenic condition of these variants had been repeatedly confirmed by frequent microscopic examinations and by heating heavy broth suspensions from agar slants and from petri dish colonies up to 85°C. for ten minutes.

The present observations confirm the views that bacteria may vary *independently* with respect to any single character. It also shows clearly the close relation of dissociation to the cytology of the cell.

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EVIDENCE ON THE SPECIFICITY OF HEXOSIDASES

A COMPARISON OF THE ACTIVITY OF ESCHERICHIA COLI AND ESCHERICHIA COMMUNIOR

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The ability of lower plants to utilize carbohydrates, which has been useful to bacteriologists in studies on the systematic relationships of bacteria, now finds a place in the work of those biochemists who are interested in the structure of various fermentable sugars. A considerable literature has grown up around the efforts of Weidenhagen to utilize biological data in substantiating his classification of enzymes, since confusing results have been obtained; and in one instance two reputable workers in different laboratories who used the same material reported opposing conclusions (Karström, 1930–31; Weidenhagen, 1930).

Working with yeast, Weidenhagen has obtained evidence for his theory that an α-glucosidase will hydrolyse all α-glucoside linkages and therefore both maltose and sucrose. Karström's studies (1930) have brought to the attention of biochemists the well known fact that *Escherichia coli* can ferment maltose but not sucrose, which is an evidence of the specificity of hexosidases, and his results have been confirmed by Virtanen (1931), Myrbach (1931), and Tauber and Kleiner (1933). While trying to reproduce identical enzyme preparations for our colleagues we were led on to work which throws some light on the causes of varying results.

Since it seems agreed that the optimum pH for sucrase and maltase action is in the neighborhood of 7.0, an aseptic technique is essential if fermentation due to contaminating organisms is to

be avoided; a condition which is appreciated by the bacteriologist but which the biochemist is apt to realize less easily.

We also wished to determine whether it was possible to obtain a preparation which would hydrolyse sucrose, since Karström's method, although suitable for the production of a maltase-reactive material, might yield one which was sucrase non-reactive. Accordingly two strains of the colon bacillus were used: *Escherichia coli*, fermenting maltose but not sucrose, and *Escherichia communior*, which ferments both sugars and from which it was feasible to expect a sucrase-reactive preparation.

The bacteria were grown in pure culture with each carbohydrate to obtain a maximum of cellular material, which was treated to secure enzyme preparations similar to those used by other workers, and the activity of the preparation was tested on maltose and on sucrose. If maltase and sucrase are identical the preparations might be expected to show a similar activity; if the enzymes are specific, the preparations (in the case of *E. coli*) might react with one substrate, while in the case of *E. communior* the preparations might be reactive with both substrates or with either one.

PREPARATION OF ENZYME MATERIAL

Karström's directions (1930–31) were followed, although a smaller amount of material was used. The method consists in growing a liquid culture of the organisms plus a specific carbohydrate, concentrating the cells by centrifuging, washing with distilled water, re-concentrating, buffering with phosphate and treating with toluol.

Broth (1 per cent peptone and 0.5 per cent meat extract) with a reaction after sterilization of 7.4 to 7.6 was autoclaved in 200 cc. amounts in flasks and 15 cc. amounts in tubes. A twenty-four hour agar slant culture of the organism was used to seed the tubes, which were incubated for twenty-four hours and the contents of three tubes added to each flask. Two grams of carbohydrate were added to the flask aseptically. The pH was tested colorimetrically at intervals, as experience showed the value of adjusting the pH with NaOH when it fell below 7.0. After four to six hours incubation at 37°C. the flasks were held at 10°C. overnight.

The pH was adjusted, the cells concentrated, washed with distilled water and re-concentrated. If the supernatant fluid was negative for reducing substances the packed cells (about 0.5 cc. in volume) were rubbed up with 1 cc. toluol and left at room temperature, with a rubber stopper to prevent evaporation. This material was acid to phenol red, and if buffered it was more difficult to kill the cells; therefore K_2HPO_4 was added after the material was sterile. The toluol and cells were mixed several times daily and small amounts of toluol added. Sterility tests were made and if growth occurred microscopic examination determined the presence of Gram-negative rods.

TEST OF ENZYME ACTIVITY

Production of monose was tested by the method of Tauber and Kleiner (1932) which is useful because monosaccharides can be detected even though disaccharide is present.[1] The small quantity of material, which is such a handicap in bacteriological work, led to the use of one-tenth amounts of the reagents and material prescribed by Tauber and Kleiner.

The substrates used were a freshly prepared 1 per cent solution of maltose in sterile distilled water and a 5 per cent solution of sucrose, since such dilutions gave a final amount of disaccharide not interfering with the test for monose. For the digestion tests six sterile test tubes were used: A, B, C, D, E, and F. To A, B, and C was added 1 cc. of the maltose solution and to D, E, and F 1 cc. of the sucrose. Tubes A and D received 1 cc. of sterile distilled water and were controls for the substrates, tubes B and F received 1 cc. of the enzyme preparation and were plunged immediately into boiling water for five minutes to prepare the inactive enzyme control. It is necessary that C and F, which receive 1 cc. of the reactive enzyme, be tested for reducing substances immediately after the addition of the enzyme in order to secure a negative reaction for the records. (Control tests using enzyme and distilled water were made when there was sufficient material and no reducing substances have been found in these controls.)

[1] The reagents used in the tests were kindly donated by Dr. Henry Tauber.

Tests for reducing substances were made according to Tauber's method by using six Wassermann tubes which corresponded to the large tubes.

EFFECT OF TOLUOL

Forty-five preparations have been made, the aim being to produce from each organism identical material which would react in a consistent manner. Our early preparations had no enzyme activity but this fault was corrected when the pH was adjusted during culture growth. Some of the preparations showed contaminations and were discarded. Some showed inconsistent reactions and to explain these the rôle played by the toluol preservative must be understood.

Karström has stated that toluol inactivates the zymase complex of the colon bacillus and we have found that without toluol the monose produced in the course of fermentation cannot be detected.

Three buffered suspensions of cells were made from *E. coli* grown in maltose broth and from *E. communior* grown in maltose and sucrose broths and to each suspension was added 0.5 cc. toluol with no attempt to mix thoroughly. The suspensions of living cells gave negative reactions in the tests for monose production from maltose and sucrose substrates, but the three toluol-treated suspensions gave the following results:

I. *E. coli* grown in maltose. With the maltose substrate, a strong reaction for monose. With the sucrose substrate, no reaction.

II. *E. communior* grown in maltose. With the maltose substrate, a very strong reaction for monose. With the sucrose substrate, no reaction.

III. *E. communior* grown in sucrose. With both substrates, a very strong reaction for monose.

The first two suspensions produced a maltase but no sucrase in accordance with the results of Karström while the third suspension confirmed the results of Weidenhagen and could be used as evidence that the enzyme splitting maltose and sucrose is one

enzyme of the α-glucosidase type. The noteworthy fact is that toluol is not an inert preservative, but plays an important part in enzyme study since it changes the end products of fermentation.

But although toluol is of use in detecting the intermediate reaction and in addition is volatile and does not precipitate protein it is not an effective bactericidal agent. Viable cells were extremely persistent in some preparations and these viable cells affected the results.

A preparation of *E. communior* (sucrose broth) was not sterile after four days at room temperature in the presence of toluol. A portion was tested on the substrates and after two hours action by the suspension, which contained 5,000 viable bacteria per cubic centimeter, there was very active sucrase and no maltase. The digestion was continued for ten days at 12°C. when a second test showed the presence of both maltase and sucrase.

The original mixture showed no bacterial growth after sixteen days, and when this was tested sucrase alone was demonstrated.

Weidenhagen and Karström have also worked with dried preparations, which would seem to be a safeguard against the activity of viable cells, but we have found that the cells remain viable for an incredibly long time. Three of our preparations illustrate this, since they were made and tested in June, 1933, the remaining enzyme dried in a desiccator, and then stoppered and kept at room temperature. In June, 1934, 2 cc. sterile distilled water was added to each preparation and they were retested for activity. Two of these preparations contained viable bacteria one year after preparation and in an incubation period of one hour gave the same results as when they were originally tested. The third preparation contained no viable bacteria and was non-reactive.

Since the sterility of cultures is uncertain, the optimum time to read the reaction is at the point when enzyme reactions occur and when living cells are in the lag phase and consequently somewhat inert. Hydrolysis of maltose occurred in forty to sixty minutes, hydrolysis of sucrose was extremely rapid as a strong test for reducing substances might be obtained after a one minute exposure of enzyme to substrate. The hydrolysis of the sugars

progressed quantitatively but a qualitative change occurring after twelve hours might be attributed to the action of some viable cells.

ENZYME ACTIVITY

Using an incubation period of one to three hours and tabulating the results from 29 preparations it was found that 5 preparations were non-reactive, 14 gave maltase only, 5 gave sucrase only and 5 gave tests for both enzymes (table 1).

With *E. coli* cultures, sucrase was never obtained, the 7 preparations were all reactive for maltase whether the preparation was made from the culture grown in maltose or sucrose.

With *E. communior* cultures, both enzymes could be obtained. Using *E. communior* grown in maltose 2 preparations were non-reactive, 6 contained maltase only, 1 sucrase only, and 2 preparations both enzymes. When the organism was grown in sucrose 3 preparations were non-reactive, 1 gave sucrase only, and 4, tests for both enzymes. It was not possible to foretell whether the preparations from *E. communior* would be reactive for one or both enzymes and this must be dependent upon a variable which we have not yet identified.

In the terminology of Karström an enzyme is "constitutive" if it is produced irrespective of the medium on which the organism is grown and "adaptive" if the production is brought about in a specific medum. In this sense maltase was constitutive for *E. coli*.

The enzyme equipment of *E. communior* is more complicated and the sucrase and maltase could not be classified as definitely constitutive or adaptive. Maltase was demonstrated 11 times; in 6 cases the culture had been grown in maltose, in the other cases the culture had been grown in sucrose which would suggest that this enzyme is constitutive for *E. communior* also; on the other hand it must not be forgotten that there were 5 sucrose-grown preparations where maltase was not demonstrated. Sucrase was demonstrated 10 times, twice with a culture grown in maltose and 8 times with a culture grown in sucrose, which may indicate some dependence upon environment in the case of sucrase production.

SUMMARY

A comparison of the sucrose and maltose fermenting equipment of two related species of bacteria, *E. coli* and *E. communior*, gives further evidence on the specificity of sucrase and maltase.

TABLE 1

Summary of the activity of 29 preparations

PREPARATION NUMBER	CULTURE	GROWN IN	MALTASE	SUCRASE
3	*E. coli*	Maltose	+	0
12			+	0
17			+	0
18			+	0
39			+	0
14	*E. coli*	Sucrose	Trace	0
40			+	0
4	*E. communior*	Maltose	+	0
10			+	0
25			+	0
29			+	0
31			+	0
32			+	+
33a			0	0
34			0	0
35			0	+
38			+	0
5	*E. communior*	Sucrose	0	0
8			Trace	+
13			+	0
15			+	+
24			0	0
28			0	0
30			0	+
33			0	+
36a			0	+
36b			0	+
41			+	+
42			+	+

I. The method was suited to the production of sucrase-reactive material and maltase-reactive material.

II. No sucrase was demonstrated in any of 7 preparations from *E. coli*; although these were all maltase reactive.

III. Sucrase alone was demonstrated in 5 preparations from *E. communior* while maltase alone was demonstrated in 6 other preparations from the same organism.

IV. Toluol prevented the dissipation of reducing substances produced in the breakdown of the disaccharides.

V. Five preparations from *E. communior* contained both enzymes. It was difficult to mix toluol and cells sufficiently well to ensure the death of all living cells. Suspensions containing viable cells tended to be reactive with the substances which the living cell was capable of attacking. A dried preparation was not a safeguard against the possibility of a few viable cells.

VI. Strong sucrase action on the substrate was found in one to ten minutes incubation time. Maltase action occurred within one hour. Suspensions with masses of living cells (toluol treated) gave reactions within one hour. Toluol-treated suspensions with only a few viable cells required incubation for twelve hours or longer before both enzymes were demonstrable.[2]

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[2] We are indebted to Dr. Laura Florence, Dr. Israel Kleiner and Dr. Henry Tauber for their kind coöperation and invaluable suggestions.

THE BEHAVIOR OF THE VIRUS OF EQUINE ENCEPHALOMYELITIS ON THE CHORIOALLANTOIC MEMBRANE OF THE DEVELOPING CHICK[1]

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The use of the developing hen's egg has recently proven of value as a medium for the *in vivo* cultivation of different filtrable viruses. It has been shown by Rivers and Schwentker (1932) and Mackenzie (1933) that the undifferentiated cells of the developing embryo are more susceptible to infection than the differentiated tissues of the hatched chick. This embryonic tissue has also been used for the *in vitro* methods of cultivation. Although it was observed by Syverton, Cox and Olitsky (1933) that the virus of equine encephalomyelitis described by Meyer, Haring and Howitt (1931) can be cultivated in a medium of minced chick embryo suspended in Tyrode solution, yet chicks 1 and 2 days after hatching are refractory to the virus. It was therefore thought of interest to study the behavior of this virus upon the membranes and embryo of the developing egg.

Two strains of the virus were used, the original one isolated in 1930 by Meyer, Haring and Howitt (1931) and an eastern strain kindly sent by Dr. C. Ten Broeck from New Jersey (1933).

The technique employed for the inoculation of the developing eggs was essentially that of Woodruff and Goodpasture (1931) with only slight modifications. The fertile egg was candled after ten days' incubation, the position of the embryo marked

[1] Read before the Ninety-fourth Meeting of the American Association for the Advancement of Science held at Berkeley, California, June 18–23, 1934.

and the surface of the shell cleansed with alcohol. A small window ½-inch square was cut through the shell over the embryonic area with a dental drill fitted with a small abrasive disk. The opening was again cleansed with alcohol and sterile cotton and the shell membrane removed with aseptic technique, exposing the chorio-allantoic membrane. The opening was ringed with sterile vaseline and sealed with a sterile cover-glass. All eggs so treated were returned to the incubator until inoculated.

A 20 per cent suspension in saline of ground guinea-pig brain containing virus was used as the base for making varying dilutions. The inoculations of the eggs were made by inserting a needle through the vaseline ring under the cover-glass and dropping 0.1 cc. of the fluid on the membrane, using a 1 cc. syringe. Inoculated eggs were incubated at 38°C. for periods ranging from three hours to ten days. Infected membranes and tissues of the embryo were tested for presence of the virus by injecting guinea pigs intracerebrally with 0.3 cc. of a ground saline suspension.

The first inoculations were made with varying dilutions of the 2 viruses, ranging from 1:1,000 to 1:100,000 for the New Jersey strain and 1:50 to 1:1,000 for the Californian. The eastern strain was transferred for 3 generations from the membrane of the egg given the 1:10,000 dilution of original inoculum. A 1:1,000,000 dilution of the ground chorio-allantoic membrane was infective for a guinea pig. The western strain passed through 4 generations from the egg given the 1:1,000 dilution of original material and was found to be infective for a guinea pig in a dilution of the membrane of 1:1,000,000.

Further continuous passages of the ground membranes were made with both viruses. The eastern strain was carried through at least 8 and the western through 7 generations. It was found practical to transmit the virus from egg to egg by inoculating the ground embryonic brain. Such transmission succeeded through 4 successive passages. Control inoculations into guinea pigs have been positive for both membranes and brain material, respectively, after each instance.

The outstanding observation noted from these egg inoculations was the marked lethal effect of the virus upon the developing embryo. Whenever the virus was active, death of the chick occurred within fifteen to twenty-four hours. Control injection of guinea pigs confirmed the presence of virus in the membranes. For this reason the lethal factor was found to be useful in determining the approximate minimum lethal dose of the virus. Varying dilutions of the 20 per cent suspension of guinea pig brain were made and the end-point determined by death of the embryo. Control inoculations into guinea pigs usually ran a parallel course, although the chick embryo was more sensitive to the higher dilutions than the rodent. In one instance the New Jersey strain killed the embryo in a dilution of 1:1,000,000 but was only lethal for the guinea pig in a 1:50,000 dilution.

As a rule, the eastern strain was more virulent than the western, killing both chick and guinea pig in higher dilutions. It was noted that the incubation time of the eastern virus was often shortened from the usual three- or four-day period to two days. The activity of the western strain was increased by repeated passage.

Marked changes were noticeable in the appearance of the chorio-allantoic membrane after inoculation of active virus. The gross and microscopic pathology of the infected membranes has been reported by Covell (1934) so that it is of interest here only to note certain changes observed on the membranes of all infected eggs whether or not there was sufficient virus present to infect guinea pigs. These changes were not obtained in the membranes inoculated with sterile saline solution or with broth.

The membrane appeared thickened and edematous as early as three to six hours after inoculation. As the lesion progressed, the thickening and gelatinous edema increased, the membrane was opaque and the blood-vessels were practically obliterated. Patchy grey areas often appeared in the older lesions, while in the most severe infections these grey areas practically covered the entire surface of the membrane.

After repeated inoculation of eggs with varying dilutions of

the viruses it was found that the eastern strain in a dilution of 1:10,000 of guinea pig brain and the western in a dilution of 1:1,000 had the most consistent lethal effect on the developing embryo. These dilutions were used routinely for all further experiments.

In order to determine the time interval involved in the multiplication of the virus, its lethal effect, and its migration to the tissues and fluids of the embryo, 2 series of eggs were inoculated, 1 with each strain of virus. A dilution of 1:10,000 was used for the eastern and one of 1:1,000 for the western strain.

Two eggs inoculated with each strain, respectively, were sacrificed from each series every three hours for a twenty-four-hour period. The chorio-allantoic membranes, the amniotic fluid, the brain and the cord of the embryo were removed at each interval of time for intracerebral inoculation of guinea pigs, while tissues were also prepared for histological study. A report on the latter has been made elsewhere by Covell (1934).

From the results of these 2 series of inoculations it was possible to plot growth curves for both of the strains as shown in figures 1 and 2. The abscissae represent the number of hours after inoculation, while the ordinates show the highest dilution of the ground membrane sufficient to cause the death of the guinea pig with characteristic symptoms.

In the first series (fig. 1) with the eastern strain sufficient virus was apparently recovered from the original inoculum after three hours' incubation to cause the death of the guinea pig. Upon repetition, however, no virus was recovered from the membrane after three hours' incubation. All other tissues were negative and the embryo was active. After six hours' incubation, the chicks were alive and no virus was obtained from any tissue in any dilution. After nine hours a heavy suspension of the chorio-allantoic membrane was infective for guinea pigs but not the higher dilutions, while virus was recovered from the brain, cord and amniotic fluid of the embryo. From then on the virus was present in high dilutions of the membrane and in all of the other tissues examined. After the fifteenth hour the embryos were no longer viable. Between the ninth and the twelfth hours the

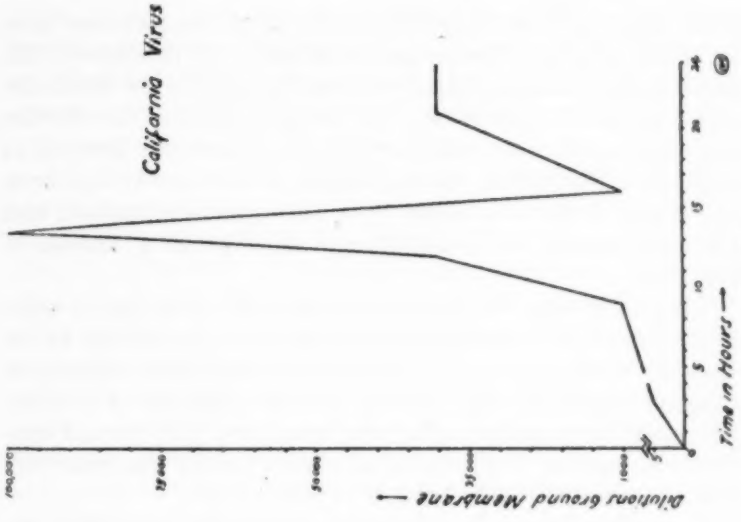
California Virus
Dilutions Brain Membrane
Time in Hours

FIG. 2

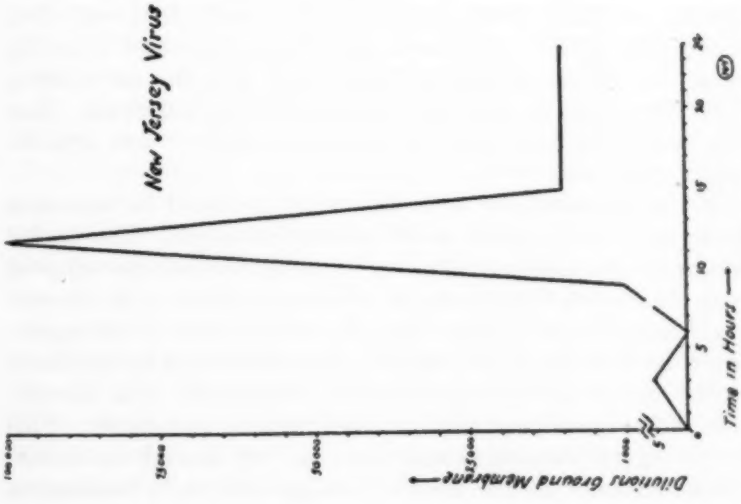
New Jersey Virus
Dilutions Brain Membrane
Time in Hours

FIG. 1

virus apparently multiplied extensively and was recovered in a 1:100,000 dilution of the ground membrane. At this interval the amniotic fluid became positive and continued so for the remainder of the period of observation. By the fifteenth hour the dilution of ground membranes sufficient to infect guinea pigs dropped to 1:10,000. Throughout the remainder of the twenty-four-hour period the chicks were dead when the eggs were opened, and the virus content of the membranes remained at a dilution of 1:10,000.

The growth curve for the western strain of virus (fig. 2) ran a similar course, no virus being recovered for the first six hours from any of the tissues examined. It was first recovered from the chorioallantoic membrane after the ninth hour in a dilution of 1:1,000. The brain, cord and amniotic fluid were also positive and continued to contain virus thereafter, except for the disappearance from the amniotic fluid during the twelfth hour.

At the latter period the virus had increased in the membrane so that a dilution of 1:30,000 killed a guinea pig. Multiplication continued until a dilution of 1:100,000 proved lethal during the thirteenth hour after inoculation. Although the virus was recovered from all of the tissues examined during these later intervals, yet the embryos remained alive until the twenty-first hour, after which death occurred. The lethal effect with this strain was therefore delayed longer than with the more active New Jersey virus. Otherwise the behavior of the 2 viruses upon the membranes and upon the developing embryo was approximately the same.

It was interesting to note that the virus could be recovered from the heart's blood of the developing chick. The pooled serum obtained after twenty-four hours from 6 embryos infected with the eastern virus produced typical symptoms upon intracerebral injection of a guinea pig. Upon repetition of the experiment the virus was recovered from the serum of embryonic chicks twelve, fifteen and twenty-one hours, respectively, after inoculation of the membranes but not after three or nine hours. This would suggest the absorption of the virus first through the chorioallantoic membrane into the blood stream and then a localization

in the nerve tissues of the developing embryo. This would further supplement the observations recently reported by one of us (Howitt, 1934) for the guinea pig, that after intranasal instillation the virus gained entrance first into the blood stream with a subsequent invasion of the nerve tissues.

It has previously been reported (Howitt, 1932) that the virus of equine encephalomyelitis was capable of being neutralized by serums of hyperimmunized animals (rabbit, guinea pig, monkey and horse). Since the present observations have shown the marked lethal effect of the virus upon the embryo of the developing chick, it was thought of interest to determine the value of the egg for use in this test.

Preliminary titrations for potency of both the western and eastern strains of virus were made by inoculating varying dilutions into both eggs and guinea pigs, respectively. The serums to be used were found capable of neutralizing the homologous virus when injected intracerebrally into guinea pigs. Equal parts of diluted virus and of immune serum were then mixed, incubated one hour at 37°C. and allowed to stand four hours at room temperature. Of each mixture 0.1 cc. was inoculated upon the membranes of duplicate eggs. Sterile saline, serum with saline alone and virus with saline were inoculated, respectively, into control eggs. Observations were made at regular intervals during the incubation, covering a period of several days.

It was found that the egg could easily and successfully be utilized as a medium for determining the neutralizing value of the immune serum. In both series of experiments performed, the eggs given homologous virus and serum remained alive, while the control embryos inoculated with virus alone died within the time limits for the dilution used. Sterile saline and the serums themselves had no lethal effect upon the developing chick.

In confirmation of neutralization tests previously noted for guinea pigs by Giltner and Shahan (1933), Ten Broeck and Merrill (1933) and Howitt (1935), it was observed that the western strain of virus was not neutralized by the eastern immune serum and *vice versa*. All the results, therefore, ran parallel with those reported when using the guinea pig. Utilization of the egg as a

quick and inexpensive medium for performing the *in vitro* neutralization test has recently been reported by Burnet and Galloway (1934) for the virus of vesicular stomatitis. It may, therefore, prove of value for those viruses which give a pronounced lethal effect upon the developing embryo.

SUMMARY

1. The virus of equine encephalomyelitis, both eastern and western strains, may be cultivated upon the chorio-allantoic membranes of the developing chick.

2. Inoculated upon the membranes of the egg, the virus may be recovered from the nerve tissues and the amniotic fluid of the embryo after a definite time interval. This progressive invasion is comparable with a growth curve ultimately leading to the death of the embryo. The virus could be recovered from the vitelline vein and from the pooled heart blood of several chick embryos after a definite incubation period.

3. Because of the rapidly lethal effect of the virus upon the embryo, the inoculation of the developing egg offers a simple and inexpensive method for titrating the potency of the virus and for carrying out the *in vitro* neutralization test.

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A NOTE ON THE SUSCEPTIBILITY OF FERRETS TO THE VIRUS OF THE COMMON COLD

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Future progress in the prevention and treatment of the common cold depends largely on the discovery of susceptible animals to which colds can be transmitted for the purpose of studying the immunity reactions elicited.

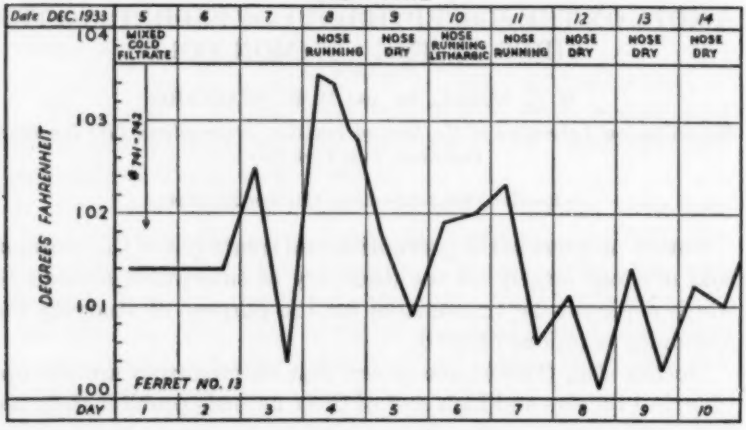
Dochez *et al.* (1930) have shown that chimpanzees are susceptible; but the size and high cost of these animals prohibit their use by most experimental workers. Smith, Andrewes, and Laidlaw (1933) found that ferrets could be infected with influenza virus, and report successful transmission with the throat washings from 5 of 8 influenza patients. They were unable to infect their animals with the virus of the common cold, although it is probably related to that of influenza. As they made but one attempt, however, it seemed desirable to us to study further the susceptibility of ferrets to the cold virus.

Nasopharyngeal washings were collected from persons with fresh colds, filtered immediately through Berkefeld "W" candles and the filtrates used to inoculate ferrets. As a rule, the filtrates of combined washings from several persons were injected into each animal, 0.5 cc. subcutaneously and 0.5 cc. intranasally. As controls, other ferrets were given sterile broth. Temperatures were noted and the animals examined twice daily for signs of respiratory disease. Every animal was kept carefully isolated for two weeks before, and during the experimental period to rule out extraneous infections of the respiratory tract. At no time did any of the stock animals exhibit symptoms of respiratory disease.

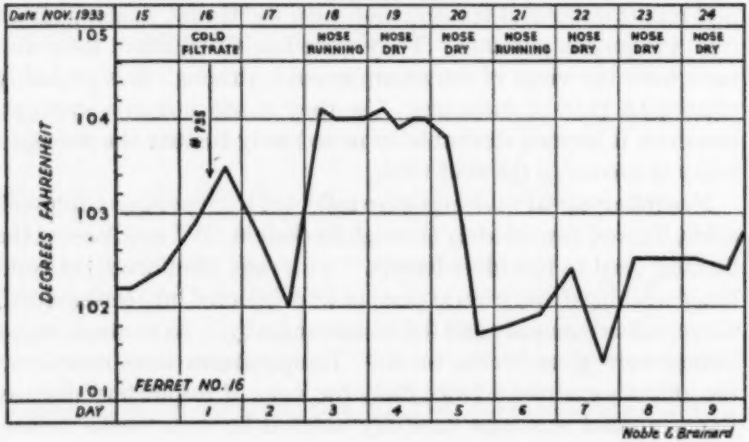
Filtrates from 40 persons with colds were injected into 12 fer-

rets; of these animals 8 were used only once and 4 were used twice, the inoculation with a second virus being given only when the

TEXT FIG. I



TEXT FIG. II



first had failed to infect, and after a rest period of seven weeks to three months. Four of the 12 ferrets developed symptoms consisting of elevation of temperature, increased nasal secretion, and

in one or two instances lacrymation and lethargy. In the temperature curve, an initial elevation appeared in twenty-four to seventy-two hours and lasted twenty-four to forty-eight hours; this was followed by a depression which lasted twenty-four to seventy-two hours. The normal temperature of our ferrets seldom exceeded 102.5°F. The maximum elevation following inoculation was 104.1°F. which lasted for two days. Rhinorrhea of mild character appeared in twenty-four to seventy-two hours and lasted about four days; the secretion was mucoid, never mucopurulent, and was sometimes sufficient to wet the hair about the nostrils. On the third or fourth day the animals were sometimes drowsy and difficult to arouse. Sample charts of 2 animals are shown.

An attempt was made to transmit the infection from ferret to ferret, and from ferret to human being, unfiltered nasal washings from an infected animal being instilled into the nose of a normal ferret and filtered washings into the human subject. The human subject, who had repeatedly handled the ferrets and had been exposed to their respiratory secretions when they sneezed or coughed on being inoculated, had never reacted to them. The second ferret in the series showed no symptoms, but the human subject, who was not isolated after inoculation, developed nasal obstruction and slight rhinorrhea in eight hours. These symptoms were mild and lasted about thirty-six hours.

It has thus been possible to produce symptoms of a mild respiratory disease in 4 of 12 ferrets by inoculation with filtered bacteria-free nasal washings from human cold cases, and apparently to transmit this condition from a ferret to a human being. The one attempt to transmit it serially from ferret to ferret was unsuccessful. The evidence presented is based on a small number of animals, but would seem to indicate that ferrets are susceptible to the virus of the common cold.

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STUDIES ON THE RESPIRATORY MECHANISM OF THE STREPTOCOCCI[1]

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There has been much uncertainty in the minds of investigators regarding the occurrence of known respiratory enzymes in the members of the Streptococcus genus. It has generally been assumed that they were absent. The present work was undertaken with the hope of clarifying the subject.

Warburg's theory (1924) assumes that any biological oxidation in which oxygen is the hydrogen acceptor is an activation of oxygen by an iron catalyst in the ferrous state. Wieland's theory (1913) states that the important process is the activation of hydrogen. Thunberg (1925) regards all biological oxidations as a series of dehydrogenations in which hydrogen is mobilized. Kluyver and Donker (1924–25), Szent-Gyorgi (1924), Fleisch (1924) and Oppenheimer (1926) have attempted to coördinate the Warburg and Wieland theories, adopting in each the part or parts that appear feasible. Keilin (1929) made a distinct contribution in demonstrating that the two theories function interdependently in the same organism, in the process of cell respiration. That cell oxidations may occur in the absence of Warburg's haemin catalysts has been demonstrated by various investigators.

In the belief that a fuller understanding of the respiratory mechanism of streptococci will eventually throw light on many questions as, for example, the mode of toxin production, and factors governing dissociation phenomena, a study was made of 22 representative strains of streptococci to determine their ability to produce respiratory enzymes.

[1] This paper covers in part the dissertation submitted to the Graduate School of Yale University by the author as a partial requirement for the Ph.D. degree.

STRAINS EMPLOYED

The twenty-two strains of streptococci and their origin are listed below. This group is comprised of eleven hemolytic, four non-hemolytic, three viridans, and four saprophytic organisms, two of which are thermophilic.

Strains of streptococci used, and sources

Serial number	*Organism*	*Source*
R-1	Hemolytic streptococcus	Scarlet fever, New Haven Hospital
R-1a	Hemolytic streptococcus	Scarlet fever, New Haven Hospital
R-2	Hemolytic streptococcus	Cellulitis, Birkhaug A 137
R-4	Hemolytic streptococcus	Mulford Laboratory No. 1045
R-5	Hemolytic streptococcus	Mulford Laboratory No. 1779
R-6	Hemolytic streptococcus	Mulford Laboratory No. 1048
R-36	Hemolytic streptococcus	Throat infection, recent isolation
R-37	Hemolytic streptococcus	Bovine udder origin, R 360
R-38	Hemolytic streptococcus	Bovine udder origin, R 359
R-39	Hemolytic streptococcus	Chicago sore throat epidemic. J. H. Brown, obtained from P. R. Edwards, × 40
R-40	Hemolytic streptococcus	Boston epidemic, J. H. Brown. Obtained from P. R. Edwards, E 41
R-11	*Streptococcus viridans*	Mulford Laboratory No. 13
R-12	*Streptococcus viridans*	Mulford Laboratory No. 14
R-13	*Streptococcus viridans*	Mulford Laboratory No. 16
R-7	Non-hemolytic streptococcus	Cardioarthritis *Small.*
R-8	Non-hemolytic streptococcus	National Type Culture Collection, Allen strain
R-9	Non-hemolytic streptococcus	National Type Culture Collection, Beattie strain
R-10	Non-hemolytic streptococcus	Mulford Laboratory No. 11
R-14	*Streptococcus liquefaciens*	National Type Culture Collection No. 799
R-15	*Streptococcus lacticus*	Laboratory culture, F strain
R-17	*Streptococcus fecalis*	J. H. Sherman
R-18	*Streptococcus bovis*	J. H. Sherman

In addition, a culture of *Escherichia coli* (strain R-21) was used as a positive control for many of the tests. The pathogenic streptococci were kept in duplicate on chocolate blood agar, one set at room temperature and the other in the ice box. The saphrophytic strains were kept on beef infusion agar.

MEDIA EMPLOYED

Since the major portion of this work was carried on with washed bacterial cells, large quantities of liquid media were utilized.

Hemolytic organisms were obtained in the largest numbers and in the shortest period of time by growing in Huntoon's (1918) hormone broth prepared without the addition of gelatin or laked blood, and containing 0.5 per cent, instead of 0.15 per cent, glucose; for the cultivation of organisms other than the hemolytic streptococci pneumococcus broth and agar prepared according to the method of Avery and Cullen (1920) was used.

CATALASE

Callow (1923), in a study of the volume of molecular oxygen produced from a weighed amount of cells, found that *Str. acidilactici*, *Str. hemolyticus*, and a cheese streptococcus formed no catalase.

McLeod and Gordon (1923) formulated their system of classification of bacteria according to catalase production and sensitiveness to hydrogen peroxide. While their work showed different sensibilities of streptococci to hydrogen peroxide, the cultures all failed to produce catalase.

Virtanen (1924), using damp masses and a dry preparation of "*Str. lactis*" under varying conditions of temperature and concentrations of H_2O_2, was unable to demonstrate catalase production.

In this investigation eighteen of the twenty-two strains of streptococci were subjected to tests for catalase. The heavy cell suspensions[2] obtained by centrifugation of broth cultures were tested for catalase by titrating with $KMnO_4$, a method evolved by Senter (1903) and modified by Tsuchihashi (1923).

The cell masses of streptococci and the uninoculated broth control both required 2.7 cc. of $KMnO_4$ to titrate the residual H_2O_2. As this is the amount of $KMnO_4$ necessary to titrate all of the H_2O_2 placed in the flasks, no catalase could have been present. The positive control organism, *Esch. coli*, on the other hand, required but 0.9 cc. to titrate the excess H_2O_2. It was concluded from these results that streptococci do not produce catalase, which is in harmony with the observations of the above-mentioned observers.

[2] The cell suspensions used in practically all of the tests made in this investigation were washed 2 to 3 times with saline solution and then vigorously aerated for one and one-half hours to remove any easily oxidizable organic matter.

PEROXIDASE

The peroxidase enzyme, like catalase, has received but brief consideration, in so far as the streptococci are concerned.

Stapp (1924) found the one culture of streptococcus used by him to be peroxidase-negative. Callow (1926) states that the one strain of streptococcus (*Str. acidi-lactici*) included in her list of organisms was faintly positive.

Anderson (1930), in her search for accessory growth substances in hemophilic organisms, used *Str. bovis* and *Str. liquefaciens* and concluded that streptococci neither produce nor utilize peroxidase. Loele (1929), using p-phenylenediamine as a reagent for the detection of peroxidase, found the one strain of *Str. pyogenes* employed to be negative. These few references to streptococci and peroxidase production appear to constitute the literary background of this subject.

Four reagents were used in this study for the detection of so-called peroxidase in bacteria. These were tincture of guaiac, a 50.0 per cent alcoholic solution of o-tolidin, a 50.0 per cent alcoholic solution of benzidine, and a 0.1 per cent aqueous solution of 2–7 diaminofluorene-HCl.[3] One drop of these reagents was added to a mixture of 0.5 cc. of cells, 2 drops of a 3.0 per cent solution of H_2O_2 (Merck's perhydrol) and 0.5 cc. of a buffer solution, pH 4.5.[4] The appearance of the characteristic color of these four dyes in the oxidized state was considered a positive test for peroxidase. Horseradish peroxidase (aqueous extract of horse radish) was used as a positive control, and mixtures of the reagents without bacteria as negative controls.

The presence of streptococcus peroxidase was demonstrated early in this work. The color that formed tended to fade quickly with all of the dyes except the 2–7 diaminofluorene-HCl. Since these findings of peroxidase in streptococci are in opposition to the results of previous investigators, a series of tests was made to

[3] Schmidt and Hinderer (1932) recently described this new reagent, which is a derivative of fluorene and phenanthren and has a chemical *structure similar* to the customary benzidine reagent.

[4] This buffer was prepared by using M/5 sodium hydroxide and M/5 potassium *acid phthalate.*

observe the influence of such factors as pH, concentration of bacterial cells, reducing intensity of the cells, and other more or less variable components of the test. The four peroxidase reagents were employed here in order to determine their relative sensitiveness as indicators of the presence of peroxidase.

pH range of the four dyes. Buffers were prepared according to Clark's (1928) method, using salt combinations to obtain a range between pH 3.0 and 8.8. The hydrogen ion concentrations were checked colorimetrically, and were correct within 0.2 pH, according to the indicator used. Employing R-37 as a test strain (before and after heating to boiling), color production was noted over the pH range, as will be seen in table 1.

TABLE 1

Showing the pH range for the four chemicals used in the detection of peroxidase

pH	GUAIAC	BENZIDINE	o-tolidin	2-7 DIAMINOFLUORENE-HCl
3.0	−	±	++	−
4.0	+	+++	+++	++
5.2	+++	+++	++	+++
6.1	++	±	++	+++
6.9	−	−	±	+
8.8	−	−	−	−

− indicates no color formation, ± doubtful, + definite color, ++ and +++ increasing density of color formed.

It will be noted from table 1 that a pH of 4.5 is a fair optimum for all reagents. This buffer strength was subsequently used for all tests. The results were the same for heated and unheated cells.

Sensitiveness of the reagents. To determine the sensitiveness of the four reagents, four cell suspensions were made up to various $BaSO_4$ turbidity standards and tested for the formation of color within a fifteen-minute period. In this experiment the dye was the only variable, the cell concentration, buffer, and H_2O_2 being identical. The results shown in table 2 are characteristic for the four color indicators employed.

Table 2 shows the sensitiveness of the four dyes that were used to detect peroxidase. It is evident that the 2–7 diaminofluorene-

HCl salt is far more sensitive than any of the other three dyes, and that it can be oxidized by one-third to one-fifth of the number of cells necessary to oxidize the others. This extreme sensitiveness is in agreement with the original claims of Schmidt and Hinderer (1932) for this chemical, when used in the detection of blood. o-tolidin at times appears more sensitive than benzidine and guaiac, although it will be shown later that the oxidation of this reagent is inhibited more by a reducing mechanism of the cell than any of the other reagents.

Time elapsing before the appearance of a positive color. Another factor to be considered in making the peroxidase test is the time of exposure. If the reducing action of the cell is very intense it may completely inhibit the oxidation of the color reagent (forma-

TABLE 2

Showing differences in sensitivity of the four reagents used for the detection of peroxidase, under identical conditions

	CELL CONCENTRATION*						HORSE-RADISH CONTROL	REAGENTS CONTROL
	1	2	3	3.5	4	6		
Guaiac	−	−	−	−	±	+	++	−
Benzidine	−	−	±	±	+	++	++	−
o-tolidin	±	±	+	++	++	+++	++	−
2-7 diaminofluorene-HCl	+	+	++	++	++	++	++	−

* Strain R-1 employed.

tion of color), or mask it for a long time. The necessary time which should elapse without color formation before the culture can be called negative is, therefore, important. The time required for the positive color to appear was determined for both living and killed (heated) cells.

Washed aerated cells were divided into two lots, one of which was placed in boiling water for fifteen minutes. Horseradish peroxidase was included as a control reagent. The customary set-up for the peroxidase test was made with the heated and unheated suspensions. Table 3 presents the results obtained with four strains of streptococci; these are representative of all of the cultures used.

Table 3 clearly demonstrates the relative reducing ability of unheated and heated washed, aerated bacterial cells. The thermolabile reducing mechanism in the heated cells was apparently destroyed. The cell suspensions exert the greatest reducing action (inhibition of color formation) against o-tolidin, while the least interference is with the 2–7 diaminofluorene-HCl reagent. While the cell concentrations were not exactly alike, it is not believed that the number of viable cells differed in any degree comparable with that shown by the reduction time with the different organisms. The difference in the thermostability of bacterial peroxidase and horseradish peroxidase is evident, the heated horseradish extract failing to oxidize the dye after an hour's contact.

TABLE 3

Showing the time elapsing before the appearance of a positive peroxidase reaction with unheated and heated cells in the presence of the color reagents

STRAIN	TIME (MINUTES)							
	Guaiac		Benzidine		o-tolidin		2-7 diaminofluorene-HCl	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
R-1	10	4	6	2	18	2	4	2
R-8	12	8	6	2	25	2	2	1
R-14	30	8	30	5	30	5	4	2
R-37	9	4	6	2	18	2	2	1
Control (Horseradish)	5 sec.		10 sec.		15 sec.		5 sec.	

Action of respiratory poisons on streptococcus peroxidase. Warburg (1925) and Keilin (1929) have shown that KCN in high dilutions is a powerful inhibitor of cell respiration. Wieland and Sutter (1928) found that M/200,000 KCN inhibits the catalytic action of horseradish peroxidase to the extent of decreasing the purpurogallin number from 280 to 130.

An experiment was conducted by the writer to determine whether bacterial peroxidase is inhibited by low concentrations of respiratory poisons. The usual test for peroxidase was performed, with and without various dilutions of the inhibitive sub-

stances. Brom-acetic acid, iodo-acetic acid, sodium fluoride, arsenious acid and ethyl urethane, which apparently poison the dehydrase mechanism of certain cells, exerted no influence on the peroxidase test. Potassium cyanide and sodium pyrophosphate (so-called iron poisons) were much less active in inhibiting the bacterial peroxidase than was KCN in its action on horseradish peroxidase.

Burnet (1927) separated bacteria into two groups, according to their sensitiveness to KCN. He also showed that the organisms which produce catalase and supposedly contain Warburg's iron respiratory catalysts are inhibited by low concentrations of KCN, whereas others, including streptococci, are not inhibited unless the concentration of KCN is increased more than twenty-five times. Burnet's observations were in part confirmed by the present author in his study of the effect of KCN on 12 different strains of streptococci. Neutral solutions of KCN which were sterilized by filtration through filter candles were added directly to pneumococcus agar in varying amounts. The agar plates were streaked with broth cultures of the various streptococci, and examined after forty-eight hours incubation.

The streptococci were not markedly sensitive to the KCN when exposed under aerobic conditions, all of the 12 strains employed showing visible growth in the presence of 0.25 per cent of the reagent. In concentrations as high as 0.5 per cent it did not prevent growth in pneumococcus broth, and three out of four strains grew in the presence of 0.75 per cent.

That the growth of the streptococci, or the respiratory mechanism of these organisms as a functioning unit, is not inhibited by relatively high concentrations of KCN lends additional support to the findings that this iron poison does not interfere with the peroxidase test in streptococci except in high concentrations. These results indicate that so-called bacterial peroxidase differs from horseradish peroxidase not only in its thermostability, but also in that it contains no iron.

In an attempt to substantiate the positive qualitative findings of peroxidase by the color tests, certain quantitative procedures were employed.

Quantitative determination of peroxidase. Several procedures were followed, including the original purpurogallin method of Willstätter and Stoll (1918). Later Guthrie's (1931) test for peroxidase and Masumune's and Kodama's (1931–32) micromethod were employed.

The results obtained following the use of these tests were in the main negative. While the addition of a small amount of bacterial suspensions gave a slight increase in color production over the reagent control, it was not possible to demonstrate a linear function with increasing concentrations of cells, even when 50 cc. of a very dense suspension of living or dead cells was used. Penrose and Quastel (1930–31), using *Micrococcus lysodeikticus*, showed that peroxidase can be extracted unchanged from the intact cells by the use of lysozyme. A few preliminary experiments were conducted by the writer with cell extracts of streptococci obtained by lysis with egg-white lysozyme and with bacteriophage. The results were not conclusive.

Thermostability. The cause of the delayed positive color reaction was suspected of being a reducing oxidase in the cells. In order to follow this up and to determine the inactivation temperature, tubes containing several cubic centimeters of a suspension of various organisms were heated over a temperature range of from 45° to 70°C. for ten minutes. After heating, the usual peroxidase test was made and the time for the appearance and intensity of the color determined, using benzidine as the color reagent.

It will be seen from table 4 that the substance in the bacterial cell which is responsible for the delayed peroxidase reaction is inhibited increasingly with rise in temperature, until at 60°C. the delay before the appearance of the color is of the same duration as at higher temperatures. This indicates that a thermolabile mechanism is operative in delaying the oxidation of the dyes used in the detection of peroxidase. Such reducing action was not apparent, however, in strain R-37, which gave a positive color at all temperatures within as short an interval of time as ten seconds.

Henneberg and Wilke (1902), using a culture of *B. xylinum*,

and Callow (1926), employing several different peroxidase-producing organisms, found the bacterial peroxidase to be thermostable. Streptococcus peroxidase was found in this study to be thermostable. It withstood autoclaving for at least one-half hour at 120°C.

It must be apparent from the numerous tests made by the writer for peroxidase in streptococci that a catalytic agent is present which gives a positive color reaction with the chemicals used in the detection of peroxidase.

TABLE 4

Showing the time (in minutes) elapsing before the appearance of a positive peroxidase reaction after the cells were heated at different temperatures

STRAIN	UNHEATED	45°	50°	55°	60°	65°	70°	BOILING
R-1	7	4	3	3	1	1	1	1
R-4	7	4	3	3	2	2	2	2
R-7	7	4	2	1	1	1	1	1
R-12	5	4	2	1	1	1	1	1
R-14	30	25	20	10	2	1	1	1
R-37	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.
Horseradish	5 sec.	5 sec.	5 sec.	5 sec.	5 sec.	30 sec.	30 sec.	

CYTOCHROME

Peroxidase is believed by Keilin (1929) to be closely associated with cytochrome, and to be represented by one of the cytochrome spectroscopic bands, possibly band C. If bacterial peroxidase is actually represented by a specific band demonstrable with a spectroscope it should offer a means of corroborating the presence of peroxidase in streptococci. Keilin (1925) observed the presence of four bands in aerobic bacteria and the absence of bands in anaerobes. Yaoi and Tamiya (1929) examined a large number of bacteria for the presence of cytochrome and showed that aerobes possess four bands, facultative anaerobes, which included one culture of *Str. erysipelatis*, two, and anaerobes none. This is the only investigation of streptococci for cytochrome known to the writer. Kuhn, Hand and Florkin (1931) have shown that the active component of horseradish peroxidase is a ferroporphy-

rinic compound having a definite two-banded spectrum, at 520 and 550 mμ. The two bands in the horseradish peroxidase and the facultative anaerobes occur at the same wave lengths.

In this investigation a Zeiss microspectroscope was used. Illumination was furnished by means of a 500 watt bulb. Cell suspensions were prepared by centrifuging broth cultures and by scraping agar plates and taking up the cells in physiological saline solution. The turbidity of these suspensions was often increased until the cell concentration equaled 50 per cent. Sodium hydrosulphite (1.0 per cent solution) was added at times to the suspension under investigation, to make certain that the pigment, if present, would be in the reduced (visible) state; KCN and pyridine were also added in the hope of forming the hemochromogens which show the strongest absorption bands.

Most of the streptococci listed in this study and three strains of *Str. erysipelatis* were repeatedly examined for the presence of cytochrome. A top yeast and *B. subtilis* were used as known positive controls showing all four bands, while cultures of *Esch. coli* and *Sal. Schotmülleri* were employed as known positive facultative anaerobes showing two bands. While the four- and two-banded spectra were observed in these positive controls, no bands were seen at any time in the cultures or suspensions of streptococci.

INDOPHENOL OXIDASE

Schultz (1910) and later Kraemer (1912) demonstrated the presence of a thermolabile oxidase in bacteria. Gordon and McLeod (1928) modified the test by using paraphenylenediamine and eliminating a-naphthol. They state that streptococci do not react to the test. Bier and Reis (1930), using essentially the same technique in testing 26 different strains of streptococci, reported that some of the cultures gave this oxidase reaction. Loele (1929), in an investigation of a series of bacteria, and employing both p-phenylenediamine and indophenol blue, found that the one culture of streptococcus used gave a negative oxidase reaction.

Keilin (1929), with the use of an improved technique, successfully demonstrated the presence of a thermolabile oxidase in yeast

and in heart muscle, and has brought forth evidence to show that this oxidase is identical with Warburg's (1924) respiratory ferment.

In the present investigation eight different cultures of streptococci were tested for the presence of oxidase, Keilin's technique being used. Yeast and *B. subtilis* were employed as positive controls. No evidence was obtained of the presence of an indophenol oxidase in these streptococci.

OXYGEN UPTAKE

Callow (1924), in a study of the oxygen uptake of washed bacterial cells in buffer solution, found that the one strain of streptococcus (*Str. acidi-lactici*) included in her group of organisms took up practically no oxygen. In order to determine whether this inability to utilize molecular oxygen is characteristic of other members of the genus, oxygen uptake studies were made with the aid of the Warburg apparatus.[5]

The bacterial cells were obtained in the usual manner, that is by centrifugation of the broth cultures and washing twice with physiological salt solution. One cubic centimeter of the unaerated cells was placed in Warburg cups containing 0.25 cc. of 2N/1 NaOH and brought up to the required volume with saline solution, the amount of the diluent depending on the other substances added. Duplicate determinations were made in every instance. The manometers were read at hourly intervals. The oxygen consumption of a bacterial suspension of an hemolytic (strain R-1), a non-hemolytic (strain R-10) and a viridans culture (strain R-11) was measured over an eight-hour period. The oxygen uptake at the end of this interval was negligible. The experiment corroborates the findings of Callow with *Str. acidi-lactici.*

Keilin (1929) showed that the presence of indophenol oxidase in yeasts, as determined by the "Nadi" reagent, can be corroborated by oxygen uptake studies. The presence of this oxidase

[5] The writer wishes to express his appreciation to Mr. S. Kasdon for his valuable assistance in the use of the Warburg apparatus.

ERRATUM

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KH_2PO_4 ..... 1.5 grams

Should have been included in formula on p. 423

apparently greatly increases the amount of oxygen utilized in the presence of living cells.

An experiment was conducted to determine whether the oxygen uptake of streptococci would be greatly increased in the presence of p-phenylenediamine. For checking purposes, oxygen uptake

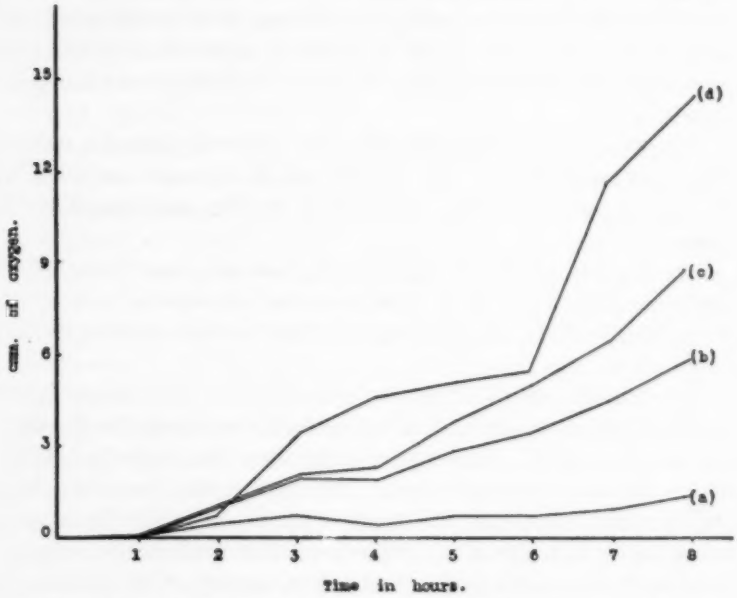

FIG. 1. SHOWING THE OXYGEN UPTAKE OF WASHED SUSPENSIONS OF STREPTOCOCCI OVER AN EIGHT-HOUR PERIOD

a, suspension of R-11 alone; *b*, suspension of R-11 plus p-phenylenediamine; *c*, suspension of R-11 plus p-phenylenediamine plus M/500 KCN; *d*, 1 cc. of p-phenylenediamine alone.

determinations were made on the bacterial suspension alone, on the suspension plus 1.0 cc. of p-phenylenediamine, on the cells plus p-phenylenediamine and 0.45 cc. M/500 KCN, and on 1.0 cc. of p-phenylenediamine solution alone. The results are shown in figure 1.

The *a* curve in figure 1 is representative of five duplicate tests.

The consumption of oxygen by streptococci, as shown here, appears negligible when compared with the large uptake of this gas reported by other investigators for other organisms.[6] It is apparent, therefore, that washed suspensions of streptococci do not possess the ability to utilize molecular oxygen in the absence of suitable metabolites. This inability to consume oxygen substantiates the negative findings in the search for indophenol-oxidase in these bacteria, an agent which is regarded as playing an important rôle in the oxygen uptake mechanism of some organisms.

The presence of p-phenylenediamine (curve *b*) caused a noticeable increase in the oxygen uptake, which increase was not hindered, but rather slightly accelerated, by the addition of KCN (curve *c*).

The oxygen uptake of p-phenylenediamine alone (curve *d*) is much greater than where the bacterial suspension was used, suggesting that the increased uptake may be due to autoxidation of this dye.

The "autoxidation" of p-phenylenediamine, when this reagent is added to bacterial suspensions, probably accounts for the oxygen uptake noted. This supports the view that indophenol oxidase is absent from streptococci. The finding that the iron poison (KCN) does not inhibit, but tends to increase slightly the oxygen taken up by this strain of streptococci, also lends added support to other findings reported in this paper, namely, that Warburg's haemin catalyst is absent from streptococci.

DEHYDRASES

Wieland (1912 to date) has held to the view that the mechanism of biological oxidation is a direct activation by the organisms of the substrate to be oxidized, rather than a process of intermediation or activation of oxygen. He considers the essential process a mobilization of hydrogen by the cell dehydrases.

Thunberg (1917) developed a technique for studying dehy-

[6] Hunt, G. A. (Jour. Bact., **26**, 341–60, 1933), using the same Warburg apparatus, showed an oxygen consumption by *Ps. pyocyanea* of approximately 275 cu. mm. of oxygen in three hours.

drases in muscle. This technique was applied to bacterial cells in an extensive series of researches by Quastel (1925) and his colleagues, and later by Kendall (1929). No studies on streptococci are known to the writer.

In applying Quastel's modification of Thunberg's technique in the present study of streptococci several changes were necessary. Methylene blue is toxic for streptococci, as was shown by Avery (1929) and others. Hence, this dye could not be used as an indicator of reduction. In my work higher dilutions of the dye were tried (up to 1/25,000) which, it was thought, might permit the use of methylene blue as a hydrogen acceptor, but these higher dilutions also were found to be toxic. Dubos (1929) showed that indigotetrasulphonate is tolerated by the streptococci. Preliminary tests made by the writer with eight strains showed that an M/1000 solution of this dye is not toxic for any of these organisms. For all subsequent tests a concentration of M/2500 was adopted, since it permitted clear-cut readings. Another modification was in the temperature, as 45°C. was found to be injurious to the organisms. In order to obtain information regarding the temperature at which cells do not proliferate, and also to determine the influence of anaerobiosis, a series of viability counts was made at various temperatures (41° to 45°C.) over a two-hour period. The counts were made by McCrady's (1915) dilution method, five tubes for each dilution being used, together with duplicate counts on blood agar plates as checks. A temperature of 43°C. appeared most practical.

Indigotetrasulphonate, Hempel desiccators in place of Thunberg tubes, and a temperature of 43.0°C. were adopted as modifications of the original technique. The set-up to determine the activating powers of streptococci on different substrates was as follows: 3 cc. of buffer, 1 cc. of indigotetrasulphonate, 1 cc. of streptococcus suspension, and 1 cc. of the particular test agent were placed in small test tubes; these were placed in desiccator jars, and the jars evacuated and incubated at 43°C.

A series of chemical substances, 101 in number, including carbohydrates, fatty, amino and other organic acids, and other substances were used to test the activating powers of the streptococci.

M/50 to M/100 solutions of these materials were prepared, adjusted to pH 7.2 with normal HCl or normal NaOH, and sterilized either by autoclaving or by filtration. Observations were made at hourly intervals to note reduction of the dye. *Esch. coli* was used as a known control, on the strength of the work of the Cambridge University investigators with this organism, while bacterial suspensions added to the buffer and dye were used as negative controls. The concentration of cells used, while varying to some extent, corresponded to a $BaSO_4$ standard of 2–4 for the hemolytic organisms, and 5–8 for the other streptococcus suspensions.

M/50 solutions of thirty carbohydrates, alcohols and glucosides were employed in these experiments. The eleven hemolytic streptococci tested showed little dehydrogenating action, with the exception of strains R-36, R-39 and R-40, the last two being cultures of *Str. epidemicus*. These were able to activate only the hexose sugars. The four non-hemolytic organisms (strains 7 to 10 inclusive) activated the same three hexose sugars, while strain R-9 acted on disaccharides and glucosides in addition. The viridans cultures (strains R-11, R-12, R-13 and R-31) activated the hexoses and quite a number of other sugars. The remaining (saprophytic) streptococci (strains R-14, R-15, R-17 and R-18) showed greater activating power in the oxidation of a larger number of substrates than the pathogenic strains. The known control, *Esch. coli* (strain R-21), revealed a wide range of activation, which is in agreement with the work of Quastel and his colleagues.

Rate of reduction. To observe the influence of the cell concentration upon the speed of reduction of the dye, six tubes were set up in which the buffer, dye and amount of glucose were constant. To these tubes was added in series 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 cc. of a bacterial suspension of strain R-12 having a turbidity equal to a $BaSO_4$ standard of 5.0. The results are given in figure 2.

Figure 2 shows that the velocity of reduction is proportional to the concentration of cells. In the presence of a suitable hydrogen donor and hydrogen acceptor a dense suspension of cells appears unnecessary.

Effect of poisons and narcotic agents on dehydrogenations. The studies of Banga, Schneider and Szent-Gyorgi (1931a) on tissue respiration show that arsenious acid influences the hydrogen activation and total respiration to the same extent. Further work by these authors (1931b) showed that iodo-acetic acid also affects the respiratory function. Lundsgaard (1932) and Meyer

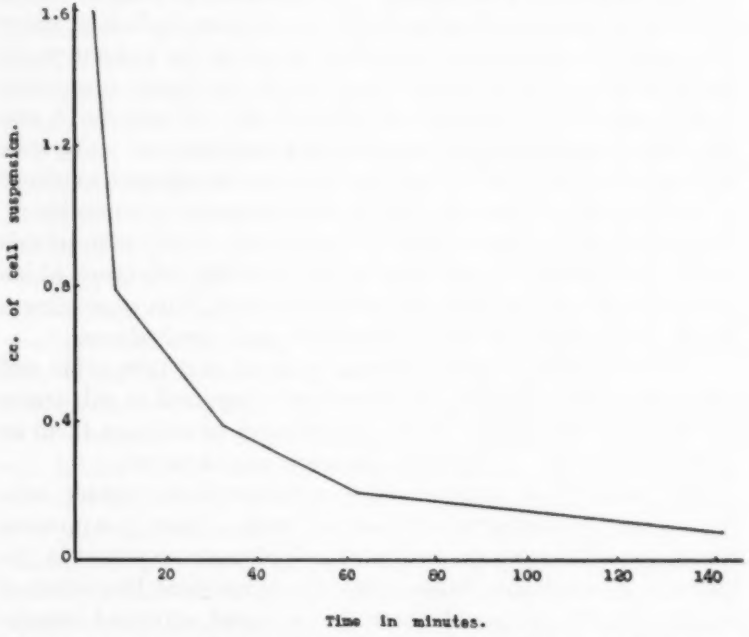

FIG. 2. SHOWING THE RATE OF REDUCTION OF INDIGO TETRASULPHONATE AS A FUNCTION OF THE CELL CONCENTRATION

(1932) further point out the specific inhibitive action of this substance.

Five different concentrations (M/10 to M/100,000) of arsenious and iodo-acetic acids and of several other respiratory or fermentative poisons, were tested for their effect on dehydrases. A mixture of bacterial suspension of strains R-7, 12 and 14, 0.5 per cent glucose as a hydrogen donor, and 1.0 cc. of each of the graded

solutions of possible inhibitors was subjected to the usual Thunberg experiment. Controls, one containing no chemical and the other no hydrogen donor, were included. The results obtained with the three different organisms were essentially the same; none of the inhibitory agents except M/10 brom-acetic and iodo-acetic acids prevented the activation of glucose by the streptococci.

Thermostability of activators. A suspension of cells of strain R-12 which had been heated for fifteen minutes in boiling water was unable to activate glucose, thus showing the enzyme to be thermolabile. Tubes containing 2 cc. of the same suspension were heated for ten minutes at 50°, 55°, 65°, 71° and 80°C. and then tested for their ability to dehydrogenate glucose. The tube that was heated to 50°C. was the only one which gave evidence of reducing action; the others showed no reduction after six hours. The inactivation temperature for dehydrases closely corresponds to the inactivation temperature of the reducing substance which is responsible for the delayed peroxidase test, thus suggesting a direct relationship between dehydrases and peroxidases.

A series of fatty, amino, dibasic, hydroxy and keto acids and other chemicals,[7] totaling 71 in number, were used as substrates to determine the ability of the streptococci to activate them as hydrogen donors. No dehydrogenation was observed.

The inability of streptococci to activate these organic substances may be accounted for in several ways. First, it is possible that these substances are activated as hydrogen acceptors in the presence of a suitable donor, with the consequent liberation of energy. Again, in accordance with the recent extended investigations into the co-enzymes of dehydrases, it appears possible that the treatment of the bacterial cells (repeated washing and aeration) may so dilute or injure the co-enzyme as to prevent action on the substrate. The rather general activating powers of streptococci on carbohydrates, as contrasted with their inability to dehydrogenate the large number of other organic substrates used here, suggest that another mechanism may be active in the

[7] For a full résumé of materials employed, tables, etc. not included in this paper readers are referred to the author's dissertation in the Yale University Library.

breakdown of these substances, or a similar mechanism with different physical properties.[8]

HYDROGEN PEROXIDE PRODUCTION

Since McLeod and Gordon's (1922) discovery that hydrogen peroxide is the cause of the bleaching of blood agar by pneumococci, much attention has been given to the question of H_2O_2 production by streptococci (McLeod and Gordon, 1923; Avery and Morgan, 1924; Valentine, 1926; Todd, 1933; and others).

Other investigations dealing with H_2O_2 production by bacteria which tend to corroborate Wieland's and Thunberg's claims that the initial and final steps in oxidations are essentially dehydrogenations are those of Bertho and Gluck (1931), Davis (1933) and Sevag (1934). They experimented with lactic acid bacteria and demonstrated that the amount of H_2O_2 formed is equivalent to the oxygen uptake. If it could be demonstrated that one of the initial products of metabolism in all streptococci is H_2O_2, then it would be possible to state that this step is essentially a mobilization of hydrogen, according to Wieland's hypothesis.

Chocolate blood agar was used for the detection of H_2O_2 in the solid medium, while the reagents 2–7 diaminfluorene-HCl and the Kastle-Meyer reagent were employed in the liquid media. An aqueous extract of horseradish was used to furnish peroxidase. The procedure was as follows: A 300 cc. Erlenmeyer flask containing 40 to 50 cc. of pneumococcus broth was inoculated with 0.1 cc. of each of the strains which previously had been subcultured twice in blood-free broth. These were tested for H_2O_2 over a twenty-one-day period. Blood agar plates were streaked frequently as a check on the purity of the cultures. An untreated flask of broth and a flask of broth containing M/28,000 H_2O_2 were carried as negative and positive controls.

The results obtained indicate that a majority of the streptococci used produce H_2O_2 in pneumococcus broth when grown in shallow layers of the medium. Peroxide was not detectable in the hemolytic organisms, under the conditions employed in these

[8] The author is at present engaged in further research dealing with dehydrases in the streptococci.

experiments, before the second to the fourth day. Two strains of *Str. viridans* (R-8 and R-9) and three saprophytic strains (R-15, R-17 and R-18) produced H_2O_2 in from four to eight hours after inoculation.

TABLE 5

Showing the production of hydrogen peroxide by streptococci when grown on different media

STRAIN	PNEUMOCOCCUS BROTH	PNEUMOCOCCUS BROTH PLUS AMMONIUM LACTATE	GLUCOSE INFUSION	CHOCOLATE AGAR
R-1	+	−	−	+
R-1a	+	+	−	+
R-2	+	−	−	+
R-4	−	−	−	+
R-5	−	−	−	−
R-6	+	+	−	+
R-7	−	−	−	−
R-8	+	+	−	+
R-9	+	+	−	+
R-10	−	−	−	−
R-11	+	+	−	+
R-12	−	−	−	−
R-13	−	−	−	−
R-14	−	−	−	−
R-17	+	+	+	+
R-18	+	+	+	+
R-21	−	+	−	−
R-36	+	−	−	+
R-37	−	−	−	−
R-38	−	+	−	+
R-39	+	+	−	+
R-40	+	+	−	+
R-15	+	+	−	+
Broth control	−	−	−	−
M/28,000 H_2O_2	+	+	+	+

In an attempt to determine if the streptococci which did not produce detectable amounts of H_2O_2, when grown in plain broth, would do so in another medium, as Gordon (1933) showed, a series of flasks containing glucose pneumococcus broth and a series containing pneumococcus broth plus 0.5 per cent ammonium lactate were inoculated and tested daily over a sixteen-day

period for the production of hydrogen peroxide. The results of these tests are compared in table 5 with those obtained in plain broth and on chocolate blood agar.

Table 5 shows the diversity of the metabolic processes of streptococci, in so far as H_2O_2 production is concerned, when individual organisms are grown on different substrates. Pneumococcus broth, pneumococcus broth plus ammonium lactate, and chocolate blood agar revealed H_2O_2 production by a majority of the strains, and with but few discrepancies. Glucose pneumo broth, on the other hand, appeared to suppress completely H_2O_2 formation by almost all of the organisms, or to interfere with the reagents used in the detection of this substance. The addition of ammonium lactate to pneumococcus broth permitted the detection of H_2O_2 in two organisms which did not produce it in plain pneumococcus broth, while four strains producing peroxide in plain broth failed to show any in this medium. The chocolate blood agar appears to be a more sensitive indicator of H_2O_2 formation than the other reagents used.

DISCUSSION

The evidence presented in this paper indicates that streptococci do not produce catalase, cytochrome or indophenol-oxidase, from which one would conclude that Warburg's haemin respiratory system is absent from this group of bacteria.

The findings of a thermostable peroxidase in these organisms does not detract from the above claim, since it is not affected by low concentrations of potassium cyanide and because it was not possible to show that bacterial peroxidase is a component of either cytochrome or of indophenol-oxidase.

The findings of Kuhn, Hand and Florkin (1931) that the active component of horseradish peroxidase is a ferroporphyrinic compound having a definite spectrum with bands at 550 and 520 $m\mu$, when contrasted with the author's failure to demonstrate bands at these wave lengths in streptococci found to contain peroxidase, suggests that the so-called peroxidases may differ from each other in certain respects. Opposed to the present author's observations are the findings of Yaoi and Tamiya (1929), who noted in one

culture of *Str. erysipelatis* the b and d bands of cytochrome, supposedly at the wave lengths mentioned above. In the present work in which repeated examinations were made of over a dozen strains of streptococci, three of which were strains of *Str. erysipelatis*, no bands were observed.

According to Keilin (1929), cytochrome is a carrier between the dehydrases and indophenol-oxidase in the systems which he studied. While it is easily conceivable that other systems and other oxidases are probably active in cell respiration, it is believed that the absence of indophenol-oxidase from streptococci brings added proof that the negative findings regarding cytochrome in this group of bacteria are correct.

Keilin (1929) evolved from his researches a scheme showing the inter-relationship between Wieland's dehydrase system and Warburg's oxidase system. In the present investigations on streptococci it has been shown that the Warburg mechanism is absent. The haemin respiratory system is present, however, in some facultative anaerobes, such as *Esch. coli* and *Sal. Schottmülleri*, while it is absent from others, including the streptococci. The lack of an iron system to complement the dehydrase system which is present in the streptococci leads one to inquire whether Warburg's haemin catalysts have a specific substitute or whether an entirely different mechanism may function in its absence.

Elliot (1932) has suggested that peroxidase may function as a counterpart of indophenol-oxidase in the presence of cytochrome, as in Keilin's scheme mentioned above. In the present investigation the reducing action of dehydrases and their inhibition of peroxidase indicates a relationship between dehydrases and peroxidases, which lends support to the present view. It is unlikely, however, that cytochrome would be present, since it and indophenol-oxidase apparently go hand in hand as an aerobic oxidase system. It is more plausible that a substitute hydrogen carrier similar to glutathione may function in a manner analagous to cytochrome, in the respiration of the type of facultative anaerobes represented by the streptococci, which were found in this study to be deficient in an aerobic oxidase system.

The question regarding the nature of the substance which acts

as a hydrogen acceptor in the streptococci must find an answer before a clearer knowledge of the mechanism of respiration in these organisms can be gained. The streptococci took up only negligible amounts of atmospheric oxygen when determinations were made in the Warburg apparatus. However, when the organisms were grown in shallow layers of broth, which permitted free aeration of the medium, H_2O_2 was detected in the majority of the cultures.

The formation of H_2O_2 in shallow layers of broth may be explained by assuming that when oxygen has reached a certain concentration it has a greater competitive affinity as a hydrogen acceptor for the mobilized hydrogen than have the other, unknown, hydrogen acceptors present in the cell or surrounding medium.

An inter-relationship between the dehydrase and peroxidase mechanisms in this investigation has been indicated. This dual system may be the complete respiratory unit in these bacteria, in which so-called iron-free bacterial peroxidase acts as a hydrogen acceptor.

SUMMARY

1. The enzyme catalase could not be demonstrated in the streptococci.

2. A thermostable peroxidase (which differed materially from plant peroxidase), was found to be present. Factors influencing its demonstration were studied.

3. The thermostable peroxidase appears to be intimately related to the thermolabile dehydrase mechanism in the cell; the latter tends to mask the peroxidase test, but is destroyed by heating.

4. Cytochrome and indophenol-oxidase (Warburg's respiratory system) could not be demonstrated in the streptococci.

5. The oxygen uptake of washed suspensions of streptococci was found to be negligible.

6. The ability of streptococci to activate 101 chemicals was studied. While it was possible to demonstrate the dehydrogenation of many carbohydrates, 71 other organic substances used in this test were not activated.

7. The respiratory mechanism of streptococci is discussed, and the conclusion drawn that the dehydrase-peroxidase system plays an important rôle in the respiration of the streptococci.

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CRYSTAL VIOLET AGAR AS A DIFFERENTIAL MEDIUM FOR STAPHYLOCOCCI[1]

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In a previous paper Chapman, Berens, Peters and Curcio (1934) showed that staphylococci could be differentiated on the basis of their hemolysis and coagulase reactions. It was further shown that a critical interpretation of the hemolysis test depended upon an accurate knowledge of the pigment production of the strain. The existence of intermediate-colored strains or of dissociants showing wide variations in color from the parent strain makes it difficult to interpret hemolysin tests of certain strains. If some correlative reaction were available, it should be useful for comparative purposes. The search for such a reaction is described in the present paper.

There was also demonstrated a correlation between the response of a strain to the reactions described and its pathogenicity for rabbits. The animal experiments were not entirely satisfactory because, in those rabbits which died, the interval between the time of inoculation and death varied considerably, suggesting that factors other than toxicity might have been responsible for some of the lethal effects. In the present study attempts were made to eliminate some of these other factors. It was hoped that this would simplify the interpretation of the animal experiments and result in an improved correlation.

Stimulated by the observation of Burbank (1929) on the effect of sodium carbonate on *Escherichia coli* and streptococci in feces, the effect of sodium carbonate on staphylococci was studied. Strains of staphylococci were treated with varying concentrations

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of this salt and transplants were made at intervals. Most strains grew from transplants taken after one hour's exposure but they failed to grow from transplants taken after three hours' exposure. Moreover, it was found that, depending upon the concentration of sodium carbonate, there was an interval when the growths from the transplants varied considerably for different strains but were characteristic for each strain. The time/concentration of carbonate necessary for the demonstration of this variable growth was somewhat critical. Essentially similar results were obtained by comparison of transplants from centrifuged carbonate supensions, indicating that carbonate carried over had little effect.

The following technic was adopted: To a series of sterile plugged test tubes were added 0.5 cc. quantities of sterile 1.0 per cent sodium carbonate (Merck reagent). A loopful of the growth from proteose lactose agar was added to each tube and thoroughly shaken. Transplants were made immediately to a solid medium to serve as controls, both of the number of bacteria present and of the effect of carbonate carried over. The tubes were placed in the incubator at 37°C. At the end of one, one and a half, two, two and a half and three hours, transplants were again taken from the carbonate suspensions and plated on the solid medium in such a way that the initial or control growth from each transplant could be compared with those obtained after incubation. After overnight incubation the growths on solid medium were critically compared and the results reported as follows: If there was a good growth from the initial transplant and from that obtained after incubation, the reaction was recorded as $++++$; if the transplant obtained after incubation showed a slightly diminished growth as compared with that from the initial growth, it was interpreted as $+++$; if there was a marked diminution of growth after incubation, it was considered a $++$ reaction; when there was no growth after incubation, as compared with an excellent initial growth, it was considered negative.

In tabulating the results to determine the correlation between the carbonate reaction and hemolysis and coagulase tests, hemolytic aureus strains (which may or may not coagulate plasma) and albus or aureus strains which coagulated citrated or oxalated

plasma were considered strains with "positive hemolysis and coagulase reactions." The best correlation was obtained with exposure to the carbonate solution for two and a half hours. However, when 151 additional strains were tested by exposure for two and a half hours, the correlation with hemolysis and coagulase reactions was only 76.8 per cent. This was considered unsatisfactory and further studies were made in an effort to find a test giving better correlation with hemolysis and coagulase reactions.

In considering the mechanism of the carbonate reaction and in an effort to obtain better correlation, it was postulated that, since the ability to grow from transplants was constant for each strain, it might have been due to factors associated with the viability of a strain or its susceptibility to the action of certain chemical agents. If this were true, it should be possible to obtain similar results by using other chemical substances. The strains were tested by the following technic:

Copper sulfate (Baker anal.), mercuric chloride (Merck reagent), crystal violet (National Aniline, comm. cert.) Merthiolate (Lilly), Metaphen (D.R.L.), ferric chloride (Eimer & Amend, T.P.), basic fuchsin (National Aniline, comm. cert.), Lysol, phenol (Merck reagent) and hexylresorcinol (S.T. 37, Sharpe & Dohme) were selected for comparative tests. These reagents were prepared in dilutions of 1:100, 1:500, 1:2,500, 1:12,500, 1:62,500, 1:312,500 and 1:1,562,000. Each dilution of the chemical substance was measured into sterile plugged tubes in 0.5 cc. quantities. Two strains of staphylococci were selected for the tests. One strain was pathogenic for rabbits, reacted positively to both hemolysis and coagulase tests and grew from the carbonate transplants (++++), while the other was nonpathogenic and reacted negatively to the *in vitro* tests. These two strains were mixed with each dilution of the chemical as was done in the carbonate reaction and transplants were made both before incubation and again at the end of one hour. Growths from the transplants were compared after overnight incubation. They were interpreted as in the carbonate reaction.

Growth curves were plotted for each chemical and they indicated that the strain which was pathogenic for rabbits and gave

positive hemolysis, coagulase and carbonate reactions could be recovered from more concentrated solutions than the strain which reacted negatively to the *in vitro* and *in vivo* tests (fig. 1).

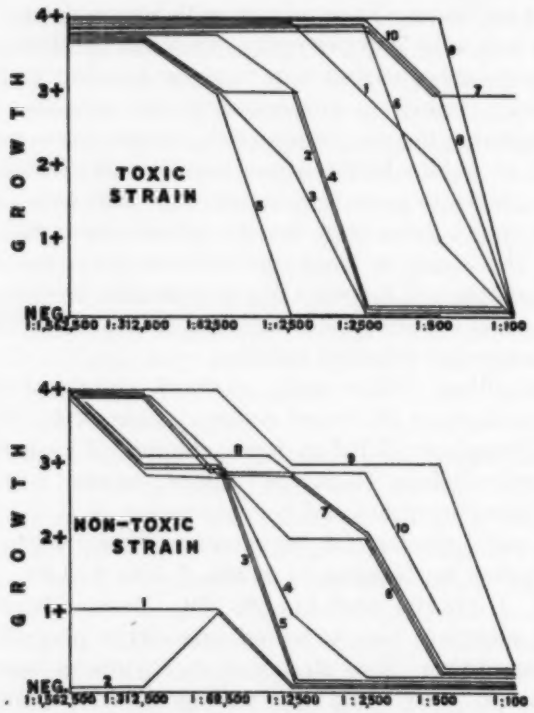

FIG. 1. EFFECT OF VARIOUS DILUTIONS OF DIFFERENT CHEMICALS ON A TOXIC AND A NON-TOXIC STRAIN OF STAPHYLOCOCCUS

One hour's exposure used throughout.

Chemicals used: (1) Copper sulphate, Baker anal.; (2) crystal violet, National—comm. cert.; (3) mercuric chloride, Merck reagent; (4) Merthiolate; (5) Metaphen; (6) ferric chloride, Eimer & Amend—tested purity; (7) basic fuchsin, National—comm. cert.; (8) Lysol; (9) phenol, Merck reagent; (10) S.T. 37.

The best differentiation between the two strains was obtained with crystal violet, but when 49 additional strains were tested with a dilution of 1:50,000 for one hour, the correlation with hemolysis and coagulase tests was only 59.6 per cent.

In the meantime, we had reasoned that, if a solid medium containing crystal violet would inhibit the growth of strains giving negative hemolysis and coagulase reactions but would permit the growth of positively reacting strains, it should be of value for the selective isolation of such positive strains. Crystal violet (National Aniline, comm. cert.) was added to proteose lactose agar in a concentration of 1 part dye substance in 100,000 parts of medium. Several strains were plated on this medium but the growths were poor. The concentration of dye was reduced to 1:1,000,000 (0.1 cc. of 1.0 per cent dye substance to 1 liter of medium). All strains grew fairly well when a large inoculum was used. It was noted, however, that growths from strains giving positive hemolysis and coagulase reactions had acquired a violet color. A few of the growths were golden colored, sometimes with violet fringes. These strains had produced an intense golden color on proteose lactose agar and had reacted positively to hemolysis and coagulase reactions. Strains of this type were usually highly pathogenic for rabbits.

A series of 594 strains were plated on the crystal violet agar to determine the degree of correlation between hemolysis and coagulase tests and the ability to acquire the violet color of the dye. The results are indicated in table 1. The best correlation (95.4 per cent) was obtained with strains producing white growths on violet agar. Orange colored growths gave almost as good correlation (92.2 per cent) but here the discrepancy may possibly be explained by the fact that the basis of comparison was the intense golden color (which obscured the violet reaction). Consequently it is possible that the 4 strains which gave negative hemolysis and coagulase reactions might have been "white" strains. The intermediate group of violet colored strains gave a correlation of 87.5 per cent. This type of organism is often of the albus variety and appears to be intermediate in other characters. This group requires further study. When the entire series of 594 strains was considered as a whole, there was an agreement with the hemolysis and coagulase reactions in 92.6 per cent.

In an effort to improve the accuracy of the violet reaction, other concentrations of crystal violet were tested. With concentrations

of 1:100,000 or more, the color of the medium was so intense that it was difficult to determine the color of the growths. On the other hand, with dilutions of 1:1,000,000 or more, the color of the growth was too pale to be distinguishable. The optimum concentration was 1:300,000. Proteose lactose agar containing this concentration of crystal violet will be referred to as "violet agar" and the phenomenon, called the "violet reaction." With this amount of dye, the violet colored growths were readily recognized after overnight incubation. The color was intensified after 48

TABLE 1

Correlation of growth of staphylococci on crystal violet agar 1:300,000 with hemolysis and coagulase reactions

COLOR OF GROWTH ON VIOLET AGAR	NUMBER OF STRAINS TESTED	ON BASIS OF HEMOLYSIS AND COAGULASE TESTS	NUMBER	AGREEMENT
				per cent
Orange (usually with violet fringe)	51	+	47	92.2
		0	4	
Violet	193	+	169	87.5
		0	24	
White	350	+	16	95.4
		0	334	
Total	594			92.6

hours but, with longer incubation periods or after storage in the refrigerator, even the pale growths became colored.

The effect of solutions of crystal violet on the color of suspensions of staphylococci was studied by plating strains on proteose lactose agar, washing off the growths the following day with 10.0 cc. of a 1:300,000 aqueous solution of crystal violet and pouring the suspensions into test tubes for comparison. Certain suspensions showed an immediate change of color and the sediments in these tubes were of a distinctly reddish hue. When the tubes were arranged according to the violet agar reactions of the organisms, the following correlation was noted. Those strains which produced white colonies on violet agar tended to bleach the aqueous

solutions and the suspensions, which settled rapidly, were blue. Suspensions from strains giving violet growths were violet, with a reddish tinge. The phenomenon is probably similar to that responsible for differential staining as described by Tolstoouhov (1929).

It was possible that some related dye might have given a sharper differentiation. When malachite green and brilliant green solutions were tested color changes occurred, but there appeared to be no correlation between the type of color change and the results of hemolysis and coagulase tests. Eosin, methylene blue, acid fuchsin, basic fuchsin, methyl green and neutral acriflavine gave results of doubtful value.

For purposes of classification, those strains which produced violet colored growths on violet agar were called "violet" strains; those which produced white growths were called "white" strains; while those strains which produced pale blue or pale violet growths were called "doubtful."

In comparing the violet reactions of staphylococcal strains with their pathogenicity for rabbits, experiments were undertaken using filtered killed suspensions in place of live unfiltered cultures. It was hoped by this method to secure more clear cut results than had been obtained previously (Chapman *et al.*, 1934).

Preliminary experiments showed that the lethal effect of whole killed cultures for rabbits varied considerably. However, most of the strains which killed rabbits when whole live cultures were injected intravenously, also killed rabbits when suitable concentrations of killed cultures were injected intravenously. Only animals weighing 1500 $\pm$ 75 grams were used in the animal experiments.

The suspensions for rabbit inoculation were prepared in as concentrated a form as possible in sterile distilled water. Serial dilutions were plated to determine the number of bacteria present. The suspensions were then treated with 1.0 per cent phenol and allowed to stand for several days in the refrigerator. Since viable organisms were still recovered, a stronger antiseptic, such as Merthiolate, was substituted. It was necessary to use a concentration of 1.0 per cent, buffered with 1.4 per cent sodium borate

to obtain sterile suspensions. After the suspensions were sterile, they were filtered through sterile cotton and injected intravenously into rabbits using 1.0 cc. quantities. Lethal effects were obtained in only a few rabbits and the inoculum was increased to 5.0 cc. Apparently the Merthiolate itself had no

TABLE 2

Correlation of hemolysis and coagulase reactions and color of growth on crystal violet agar 1:300,000 with lethal effect of 5.0 cc. of Merthiolated cultures of staphylococci for rabbits when injected intravenously

Results obtained with strains producing white or pale growths on crystal violet agar.

STRAIN NUMBER	COLOR	HEMOLYSIS	COAGULASE	VIOLET AGAR	NUMBER PER CUBIC CENTIMETER (PLATED)	EFFECT ON RABBIT	POSSIBLE ERROR
20	Albus	0	0	White	7.2×10^{12}	1. Died 12 hours 2. Survived* 3. Survived	Rabbit
24	Albus	0	0	Doubtful		Survived	
6194	Albus	0	0	White		Survived	
8	Albus	0	0	White		Survived	
6209	Albus	++++	0	White		Survived	
6195	Albus	++++	0	Doubtful		Survived	
6183	Albus	+++	0	White		Survived	
18	Albus	+++	0	White		Survived	
11	Albus	0	0	White		Survived	
2	Albus	0	0	White		Survived	
117	Albus	+	+	White	3.5×10^{10}	Survived	Coagulase
121	Albus	0	0	White	1.0×10^{10}	Survived	
126	Albus	+	0	Doubtful	2.2×10^{10}	Survived	
130	Albus	0	0	White	2.8×10^{8}	Survived	
139	Albus	++	0	White	3.0×10^{7}	Survived	
143	Albus	0	0	White	1.0×10^{7}	Survived	

* Survived four days.

lethal action under the conditions of these experiments because, even when a volume as large as 5.0 cc. was injected, no effect was noted in the test animals, provided strains considered nonpathogenic on the basis of the injection of live cultures were used.

Using the technic outlined, 28 strains were injected into 48 rabbits. With a few exceptions, which will be discussed later,

those strains with negative reactions did not produce death, while those with positive reactions produced lethal effects in rabbits.

In considering the correlation of the violet reaction with the lethal effect of the killed suspensions for rabbits it was noted that, with the exception of one rabbit, in which case the effect was probably an error, "white" or "doubtful" strains did not kill

TABLE 3

Correlation of hemolysis and coagulase reactions and color of growth on crystal violet agar 1:300,000 with lethal effect of 5.0 cc. of Merthiolated cultures of staphylococci for rabbits when injected intravenously

Results obtained with strains producing violet or golden growths on crystal violet agar.

STRAIN NUMBER	COLOR	HEMOLYSIS	COAGULASE	VIOLET AGAR	NUMBER PER CUBIC CENTIMETER (PLATED)	EFFECT ON RABBITS	POSSIBLE ERROR
1	Albus	++	++++	Violet		Died overnight	
15	Aureus	++++	+++	Violet		Died overnight	
22	Aureus	++++	+++	Violet		Died 24 hours	
25	Albus	++++	0	Violet		Died 36 hours	Coagulase?
124	Albus	+	+++	Violet	2.3×10^{10}	Died overnight	
133	Aureus	++++	+++	Golden	4.8×10^{10}	Died overnight	
138	Albus	++	+++	Violet	1.8×10^{8}	Died overnight	
145	Aureus	+	+++	Violet	6.5×10^{8}	Died overnight	
5	Aureus	0	++++	Violet	8.9×10^{10}	1. Died 36 hours 2. Died 48 hours 3. Survived	Rabbit
98	Albus	++++	0	Violet	2.4×10^{10}	Survived	Violet?
146	Aureus	++++	+	Violet	6.3×10^{14}	Died 4th day	
150	Albus	++	++++	Violet	3.8×10^{13}	Died 48 hours	

rabbits in four days (table 2). Among these strains was one which gave a positive coagulase reaction. Three of the strains with doubtful violet reactions were included in this group but they had no effect on the rabbits and should, therefore, be considered as white strains. Confirming the work reported in the previous paper (Chapman *et al.* (1934)), most of the killed cultures of hemolytic non-coagulating albus strains had no apparent effect on rabbits.

In contrast with this group of white or doubtful strains, golden or violet strains were with one exception, uniformly toxic for rabbits (table 3). Most of the animals injected died overnight, a few died in one to two days, and one did not die until the 4th day. Strain 98 failed to affect the rabbit and, since the other *in vitro* tests were negative, the violet reaction was probably in error in this instance. One of the strains (no. 25) gave negative hemolysis and coagulase reactions but the violet reaction was

TABLE 4

Summary of correlation between different tests

Methods used: Crystal violet, exposure to 1:50,000 crystal violet for one hour; sodium carbonate, exposure to 1.0 per cent Na_2CO_3 for two and one-half hours; hemolysis, hemolysis of rabbit blood agar; coagulase, coagulation of oxalated human plasma; violet agar, color of growth on 1:300,000 crystal violet agar; rabbit inoculation, intravenous inoculation of 5.0 cc. of a heavy killed suspension.

TESTS COMPARED		STRAINS TESTED	AGREEMENT
(a)	(b)		
			per cent
Crystal violet	Sodium carbonate	47	68.1
Crystal violet	Hemolysis and coagulase	47	59.6
Hemolysis and coagulase	Sodium carbonate	151	76.8
Violet agar	Sodium carbonate	101	82.2
Violet agar	Hemolysis and coagulase	1034	93.0
Violet agar	Rabbit inoculation	28	96.4
Hemolysis and coagulase	Rabbit inoculation	28	92.8
Rabbit inoculation (probable accuracy based on results with 48 rabbits)		28	91.3

positive and the rabbit died in thirty-six hours. This suggested that the violet reaction was correct and the coagulase in error.

Bacterial counts presented difficulties which raised doubts as to their accuracy and, since good correlation was obtained with crude suspensions, the strength of which was estimated from their turbidity and viscosity, it was assumed that turbidity or viscosity was a sufficiently precise method for the present purpose. The bacterial counts are recorded in tables 2 and 3.

The effects of strain 5 on rabbit 3 and of strain 20 on rabbit 1 indicate possible errors in the animal experiments. On the basis

of these experiments it would appear that the violet reaction and the hemolysis and coagulase reactions are approximately of the same order of accuracy as the animal inoculation tests.

The correlation of different reactions is summarized in table 4. Some data based on extended experiments have been included.

From these experiments it is concluded that the violet reaction may prove to be a useful addition to hemolysis and coagulase reactions for the study of staphylococci.

SUMMARY

In an effort to improve the correlation between hemolysis and coagulase reactions and animal inoculation tests, a search was made for reactions which might give correlation with hemolysis and coagulase tests, and which might be used to check their accuracy.

When strains were mixed with 1.0 per cent sodium carbonate for two and a half hours and transplants made at the end of that time, growths were obtained from certain strains. There was a 76.8 per cent correlation between the ability of a strain to grow from the carbonate transplant and the presence of hemolysis and coagulase factors. Attempts were made to improve this correlation by substituting other chemical substances for the sodium carbonate. Two strains were selected for comparative tests. One reacted positively to hemolysis and coagulase tests, grew from carbonate transplants and was pathogenic for rabbits; the other gave negative hemolysis and coagulase tests, did not grow from carbonate transplants and was not pathogenic for rabbits. When tested with decimal dilutions ranging from 1:100 to 1:1,562,000 of copper sulfate, crystal violet, mercuric chloride, Metaphen, Merthiolate, ferric chloride, basic fuchsin, Lysol, phenol and hexylresorcinol, growths were obtained from more concentrated solutions of each chemical when the positively reacting strain was used, as compared with similar tests using the negatively reacting strain. The greatest differentiation was obtained with crystal violet. However, exposure of a number of strains to solutions of crystal violet failed to correlate with hemolysis and coagulase reactions. When crystal violet was added

to solid media, it was noted that strains reacting positively to hemolysis and coagulase tests produced violet growths, or golden growths tinged with violet, while those strains which reacted negatively to hemolysis and coagulase tests produced white or pale growths. The correlation between hemolysis and coagulase tests and color on crystal violet agar was 93.0 per cent, on the basis of tests on 1034 strains.

The results of violet agar and hemolysis and coagulase reactions were compared with animal inoculation tests using 5.0 cc. of dense Merthiolated suspensions and the following correlations noted: (1) Between violet agar reactions and toxicity for rabbits, the correlation was 96.4 per cent; (2) there was a correlation of 92.8 per cent between hemolysis and coagulase reactions and the animal inoculation tests. On the basis of 48 tests on 28 strains, the animal inoculation tests were probably correct in 91.3 per cent of the rabbits. This would place hemolysis and coagulase reactions, the violet agar reaction and rabbit inoculation of Merthiolated cultures in the same order of accuracy.

CONCLUSION

The color of growths of staphylococci on crystal violet agar 1:300,000 is a valuable characteristic for the differentiation of staphylococci.

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CHITINOVOROUS BACTERIA[1]

A PRELIMINARY SURVEY

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Chitin, the flexible but tough insoluble substance which reinforces the cell walls of the fungi and the exoskeletons of the arthropods, was probably first prepared in 1811 by Braconnot. He used the word fungine to designate the insoluble nitrogenous residue obtained by heating mushrooms with alkali. It is now known to be the characteristic framework substance of the fungi, though thus far all attempts to isolate it from the true bacteria have failed. It was first called chitin in 1823 by Odier who got it from the wing covers of May beetles. It occurs in most of the invertebrates, lobster and crab shells being the most convenient source. It was not until about the turn of the century that fungine and chitin were recognized as nearly if not exactly identical; possible physical or chemical differences between animal and vegetable chitin have been the subject of research up to the present day.

Considering the large amounts of this substance constantly built up and broken down in nature, surprisingly little work has been done on the organisms capable of attacking it. So far as is known, the higher animals do not digest it. Abderhalden (1911) calls attention to the fact that the thinning of chitinous shells in the digestive tracts of selachians and fishes is due to the acidity of the gastric juice—the chitin is not destroyed and can be recovered from the lower intestine. Apparently no one has

[1] The work here summarized was done in the Department of Bacteriology and Immunology at the University of Minnesota, Minneapolis, Minnesota, and submitted to the Faculty of the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

considered the possible availability of this compound as a source of carbon, nitrogen or energy to animals. Cellulose, another relatively insoluble polysaccharide, is a source of energy to herbivores after it has been hydrolysed by their intestinal bacteria. Whether chitinovorous bacteria perform an analogous function in the digestive tract of any animal is unknown.

Of even greater importance is the rôle of chitin in the metabolism of microörganisms and consequently in the nitrogen and carbon cycles, both of soil and of water. Evidence has come to light within the past few years that soils contain numerous types undiscovered by pioneer bacteriologists. The cellulose bacteria described by Winogradsky (1929) are morphologically and culturally very different from those previously recognized, and it seems reasonable that modern elective culture methods might yield organisms capable of breaking down other complex organic substances. The, as yet, unpublished work of Henrici on the ecology of water organisms reveals a bewildering variety of types never before investigated. The rigid, relatively insoluble chitinous structures which are constantly disintegrating in soil and water, must obviously be susceptible of attack by microörganisms of some kind.

Zopf (1880) reported that certain fungi parasitic on insects can digest chitin. Benecke (1905) was the first to describe a bacterium which used chitin as a food. He prepared chitin from crabs, added it to water containing 0.03 per cent K_2HPO_4, 0.03 per cent $MgSO_4$, and 1.5 per cent NaCl, and inoculated this medium with rotting plankton from the Keil harbor. After several transfers he isolated an organism which he named *Bacillus chitinovorus*, aerobic, gram-negative, asporogenous, rod-shaped, motile with peritrichous flagella, often aggregated into zoögleae, occasionally in chains. It broke down the chitin without producing any acidity, odor or characteristic end-products. Neither glucosamine nor any other reducing substances appeared in the cultures; the intermediate products of chitinolysis must have been consumed as soon as they were formed. The organism produced acid from glucose and sucrose, and could use lactose as a source of energy though no mention is

made of acid production from the latter. Gelatin was liquefied and nitrate reduced, but starch and cellulose were not attacked. From rotting basidiomycetes he also isolated bacteria which grew well on crab chitin, but he did not describe or name them.

Folpmers (1921) sixteen years later isolated two strains of chitinovorous bacteria from the harbor water at Kiel; one he considered identical with Benecke's organism, the other differed from it only in its inability to attack gelatin. He made the interesting observation that butyric or butylic bacteria added to his cultures caused the chitin to be broken down twice as fast and that gas and butyric and acetic acids were formed by these mixed cultures. He also stated that in the soil chitin is destroyed by bacteria and by actinomycetes, but he did not describe any pure cultures.

Steiner (1931) reports that mixed cultures from the water and mud of an Alpine lake attacked chitin both aerobically and anaerobically. No pure cultures were isolated.

Rammelberg (1931) prepared chitins from fungi and from crabs, and, after having shown that there was little or no chemical difference between them, used each as the sole source of energy in an otherwise mineral medium, which he inoculated with material from a compost heap. The same organism was isolated from both media, and the pure cultures obtained by plating grew equally well on chitin from either source. In practically all the biological characteristics that were determined this organism resembled *B. chitinovorus*. Rammelberg describes in considerable detail a cycle of morphological development which includes rod-forms, filaments, large ovoid and signet-ring forms and symplasms, and emphasizes the fact that Benecke also observed large oval cells as well as bacilli.

Stormer (1908) and Jensen (1932) have both reported chitin destruction by actinomycetes in soil, and Johnson (1931, 1932) isolated and described various myxococci which grew on crab or fungus chitin.

Benecke's organism and the two variants reported by Folpmers and by Rammelberg are apparently the only chitinovorous Eubacteriales thus far described. Consideration of the vast

amount of complex chitin-bearing materials constantly being broken down in a variety of situations in the sea, in fresh water, and on dry land, makes it seem improbable that the ability to elaborate a chitinase should be confined to a single species among the true bacteria. The question also arises, whether the chitinovorous bacteria form a highly specialized, well defined group, or whether chitin destruction in nature is due to ordinary soil or water organisms whose activities in that direction have never been observed. The very real theoretical importance of chitin as a possible source of energy for heterotrophic microörganisms, and as a link in the carbon and nitrogen cycles suggests and justifies further investigation. The work to be described has for its object the isolation of chitinovorous bacteria from a variety of habitats, a study of their morphological and cultural characters and some comparison of these cultures with others previously described.

PROCEDURE

Chitin was prepared as follows: Crab shells were scrubbed as free as possible of flesh and dirt and decalcified in cold one per cent hydrochloric acid, which was changed three times within a period of about a week. The limp leathery shells were washed, cut into pieces of suitable size and shape, and soaked in 2 per cent potassium hydroxide for ten days, during which period they were stirred several times and on three occasions brought to a temperature just below boiling and then allowed to cool. This should remove the protein and other organic matter except chitin; most of the pigment dissolves out in the alkali. As traces of pigment would not render the product unsuitable for this work, while any chemical alteration of the chitin itself should be avoided for theoretical reasons, the often recommended complete bleaching by permanganate and bisulphite was omitted. The material was washed free of alkali, extracted three times in boiling ethyl alcohol, and dried—after which treatment it appears colorless. For purposes of comparison, a small supply of lobster chitin was procured from the Eastman Company. This, according to their statement, was prepared by the same method, except

that the shells were coarsely ground after decalcification and the alcohol extraction was probably more thorough—it was continued "until a day's extraction failed to bring out any solid on evaporation of a portion of the extract."

The home-made crab chitin offered certain practical advantages. In the enrichment and cultivation media it is always advisable, and in the case of some strains actually necessary, that the chitin protrude above the liquid, as the most vigorous growth and the first visible disintegration of the chitin takes place usually at the surface. A strip about 5 to 7 mm. wide and 4 to 5 cm. long placed in a test tube containing about 5 cc. of liquid meets this requirement admirably and comparisons of amount of turbidity and presence of a pellicle or sediment are much easier when the liquid is not filled with a mass of small irregular bits. Moreover, the beginning of chitin destruction is much more easily observed on a piece with a straight entire edge. Crab shells can be bought in the market and are convenient to handle; a number of strips can be cut from each one, and the remaining portions ground fine and used in the plating medium.

The saline fluid used as a basis in all cultures was distilled water containing 0.03 per cent each of K_2HPO_4, $MgSO_4 \cdot 7H_2O$ and NaCl. The tubes used for enrichment and transfer of mixed cultures and for isolation of colonies fished from plates, each contained a strip of chitin nearly covered with the fluid. After autoclave sterilization the pH was 7.6 to 7.8. Obviously any organism which can grow in pure culture in such a medium must derive its energy and carbon from the chitin and also its nitrogen unless it is able to fix this element from the air. A useful variation is the addition of a trace of peptone. In the case of most of the organisms studied, this hastens the growth of pure cultures. It is not advisable to use it for isolations from plates, however, as it tends to favor the persistence of organisms devoid of chitinase. Apparently these almost always accompany the true chitin destroyers, and mixed colonies are the rule rather than the exception on the first platings.

For obvious reasons, the ordinary nutrient agar and gelatin plates used in Rammelberg and Benecke's method of strewing

sterilized ground chitin over the surface of plates, are far from ideal. A plating medium containing finely ground chitin evenly dispersed through a 3 per cent solution of agar in the basic saline solution has proved entirely satisfactory. A dissecting microscope is almost a necessity for fishing colonies, as they are often too small to be seen well with the naked eye, particularly when obscured by chitin particles.

In general, the material to be investigated was inoculated into the simple enrichment medium, transferred to a second tube as soon as growth was evident (usually within ten days if at all), then to a third tube, and then plated out. Further transfers before plating were apparently of no advantage. Representative colonies were fished, incubated and replated until the plates appeared to contain but one type of colony. Transplants from such homogeneous plates were regarded as pure cultures unless more than one morphological type appeared in stained smears. Single cell isolation seems superfluous in this type of work.

Comparison of successive sets of plates often suggested symbiotic relationships in the original mixed cultures. Sometimes the colonies on a crowded plate were larger than those of the next dilution which were well spaced. In a few instances intermediate mixed cultures in which the chitin had noticeably disintegrated showed, on plating, two or more kinds of colonies which grew well together on the same plate, but no one of which would grow alone in the simple enrichment medium. Limitations of time and equipment have prevented the study of these possibly associative types and of the companion types helpful to the true chitinovorous strains. Also, there is always the possibility that there may be organisms capable of attacking chitin only in the presence of an inorganic source of nitrogen. If there were any in the materials investigated, they were missed by this procedure.

Brilliantly colored colonies were numerous on the first plates in the majority of cases. They were usually mixtures of a colorless chitin destroyer and a chromogen which was unable to persist in pure culture on these media. This was true of many beautiful pink and red colonies, most of the green and fluorescent types,

and a very interesting small vibrio which grew in tiny navy-blue colonies in the vicinity of truly chitinovorous colorless ones. Actinomycetes were numerous and varied, and grew luxuriantly on the chitin though they apparently used it very economically as the strips did not disintegrate to any visible extent until months had elapsed. In view of the present status of the taxonomy of the actinomycetes, no attempt has been made to classify them. Turquoise-blue, flesh-pink, salmon, rose, mulberry, green, yellow, brown, and black varieties have been isolated. Molds appeared less often than actinomycetes. They were all discarded.

TYPES ISOLATED

Chitinovorous eubacteriales were isolated with comparative ease from a great variety of sources. About 250 pure cultures have been studied thus far, and classified according to their morphological and biochemical characters, as tabulated (Table I). They are obviously not members of any single species or genus, but rather fall into a number of fairly well-defined types, most of which do not resemble any of the species listed in Bergey's *Manual* (1934). Some were isolated from but one of the sources investigated, others from so many as to make it appear that they are widespread in nature. Fifty or more pure strains are not included in this report because the data are not yet complete; some of them at least are different from any of the types about to be described. It seems likely that as more sources are investigated still more types will be discovered.

Type I (fig. 1) was isolated repeatedly from the water, bottom mud and plankton of Lake Alexander over a period of two months, and also from the mud of Lake Minnetonka, from the intestines of a frog, and from the intestines of 5 bats. The 52 cultures studied attack chitin very vigorously, are strikingly similar, and apparently belong to a well-defined species. The heavy pink growth on potato, often with brownish gas blisters, is extremely distinctive. Hanging drops sometimes exhibit an entertaining peculiarity of behavior. Among the small actively motile bacilli one sees what appear at first glance to be large flexible

TABLE 1

Morphological and cultural characteristics of types

TYPE	GRAM STAIN*	SHAPE	FLAGELLA†	MILK	GELATIN	INDOL	POTATO	GLUCOSE	MALTOSE	MANNITOL	SUCROSE	LACTOSE	STARCH	CELLULOSE‡	NITRATE REDUCTION	PIGMENTATION
I	−	Plump rods	Polar	Neutral, peptonized	+	+	Abundant, pink	AG	AG	AG	AG	−	+	−	+	
II	−	Plump rods	Polar	Neutral, peptonized	+	+	Abundant, putty-color	AG	AG	AG	AG	−	+	−	+	
III	−	Plump rods	Peritrichic	Alkaline, peptonized	+	−	Colorless	AG	AG	AG	AG	−	−	−	+	
IV	−	Rods	Polar	Peptonized	+	+	Colorless	A	A	A	A	−	+	−	+	
V	−	Rods	Peritrichic	Peptonized	+	−	Colorless	A	A	A	A	−	−	−	+	
VI	−	Vibrios	Polar	Slightly alkaline ?	0	−	0	A	A	−	−	−	−	−	+?	
VII	−	Plump rods	Polar	Alkaline	+	−	Brownish	A	A	−	−	−	−	−	+	
VIII	−	Plump rods	Polar	Peptonized	+	+	Slight growth	A	A	A	−	−	+	−	+	
IX	−	Slender tapering rods	Polar	No change	+?	−	0	0	A	0	A	A	−	−	−	
X	−	Delicate curved rods	Polar	No change	0	0	0	−	−	−	−	−	+	+	−	Yellow on cellulose and starch
XI	−	Straight rods	Motile	Alkaline, peptonized	+	−	Brownish	−	−	−	−	−	−	+	+	
XII	−	Pleomorphic	Polar	Sometimes peptonized	+	−	Blackened, growth yellow	−	−	−	−	−	+	−?	−	Yellow and "tyrosinase"
XIII	−	Pleomorphic	Polar	No change	+?	−	0	−	−	−	−	−	+	−?	+	Yellow and orange on chitin
XIV	−	Small rods	Polar	Alkaline, peptonized	+	−	Brown	−	−	−	−	−	−	−	−	Green, fluorescent
XV	−	Rods	Polar	Alkaline, peptonized	−	−	Yellow	−	−	−	−	−	±	−	−	Pink on chitin only
XVI	+	Rods with square ends	Motile	No change	+	−	Darkened	A	A	A	A	−	−	−	+	
XVII	+	Pleomorphic	Motile	Slightly alkaline	−	−	Eggshell pink	A	A	−	−	−	−	−	+	
Bc	−	Rods	Peritrichic		+			A (G?)			A (G?)		−	−	+	

Bc = *B. chitinovorus Benecke*; 0 = no growth; A = acid; AG = acid and gas; and ± = some strains +, others −.

* Kopeloff and Beerman modification.

† Gray's stain.

‡ Skinner's medium.

granular organisms, writhing and bending. Close scrutiny reveals that these are groups of the small rods, lying side by side in what might be called a "palisade-chain" arrangement. At this stage of the culture's development, there seems to be a definite tendency for actively motile rods which come into close contact to snap together side by side—an individual arriving against a chain does not become attached except at the end. Sometimes a group of 3 or 4 members becomes detached from the end of a chain, and moves off as an independent unit which may take on recruits at either end, or snap into place at the end of some other chain. The whole picture is absurdly suggestive of an attempt at military drill in the midst of a restless crowd.

Nineteen other cultures from the water, bottom mud and plankton of Lake Alexander comprise type II. They differ from type I only in the color of their growth on potato, which is rather yellowish at first but later takes on a neutral putty shade. The "palisade chains" were not observed in any of this group. However, no special search was made for them, and they may occur in cultures of suitable age.

Type III was isolated from the digestive tract of a speckled trout and is decidedly different from the first two types (fig. 2). On liquids it grows in a thick white layer at the surface, which is somewhat slimy, as is the growth on solid media.

These three types all produce acetyl-methyl-carbinol from glucose, but fail to attack lactose. They obviously do not belong to any of the well-recognized species. Type III might possibly be included in the genus Proteus. Types I and II are excluded from that genus by their morphology and from Achromobacter by their active gassy fermentations.

Type IV, which attacks the same sugars, but without gas formation, was isolated from crayfish, from decaying May-fly nymph shells, from the intestines of snipes and of bats, and from sand at the edge of Lake Minnetonka. This type may be related to the genus Pseudomonas, as more than half of the strains studied have exhibited at some time a trace of fluorescent greenish pigment on agar, though never on gelatin, peptone or potato. Visible chitin destruction is not so prompt as in the first three types and

the cultures are more likely to die out after a few weeks. The somewhat greater acidity produced by these strains in the chitin tubes (terminal pH about 5) may be responsible for this fact.

Type V includes a group of cultures isolated from the bottom mud of Lake Alexander, which are peritrichic and fail to digest starch or produce indol. Otherwise they resemble type IV.

Type VI (fig. 3) appeared in tubes inoculated with material from a ravine on the University Campus; leaf mold, soil from the edge of quiet pools, and sand from beneath the rapidly running water of a little stream. They produce a markedly acid reaction in the chitin tubes, so that with Andrade's indicator the chitin strip itself takes on a deep rose color. The growth on agar slant is very delicate, transparent, and spreading. This type does not resemble any of the vibrios listed by Bergey.

From the stomachs of two different bats type VII was isolated. These are straight plump monotrichic rods similar to type VI in their reactions on carbohydrates, but more active in their growth on other media.

Type VIII, isolated from a compost heap, resembles type VII in morphology, but is even more active on culture media. These last two types could be classified as members of the genus Achromobacter, but correspond to no species now listed.

From sand now exposed by the receding water of Lake Minnetonka type IX was isolated, long tapering monotrichic gram-negative rods, with faintly staining oval forms also present in aging cultures (figs. 4 and 5). The sugar reactions were peculiar. When first isolated the organism failed to grow at all in glucose or mannitol broth, but on the disaccharides, maltose, lactose and sucrose it produced a slimy growth and a small amount of acid. After several transfers on artificial media, it now grows on glucose broth but produces little or no acid. Starch and cellulose have never been attacked.

The remaining gram-negative types are characterized by failure to produce acid from mannitol or the four sugars used. Type X is evidently a definite species and as widespread in soil and water as type I. Thirty-four pure cultures were studied, most of them from the bottom mud of Lake Alexander, some from

Lake Minnetonka, the rest from dead crayfishes, from decaying May-fly nymph shells, from cultivated sandy loam, and from compost. They are gram-negative, extremely long and narrow when first isolated, delicately curved, and motile by a single flagellum (figs. 6 and 7). The enrichment medium becomes turbid within a week and the chitin disintegrates at the water surface a few days later. On the ordinary routine media there is little or no activity. Agar slants exhibit a very characteristic, practically invisible growth. On turning the tube toward the light at precisely the proper angle one sees a very faint film on the surface, as if "G" colonies had become confluent. However, if a small amount of water is agitated over the surface of the agar, it becomes turbid and opaque. Stained smears show hundreds of the delicate graceful threads. On starch agar there is a fairly abundant shiny yellow growth and active digestion within two days. In the cellulose medium the paper is promptly covered with a thin lemon-yellow film above and below the water line, and begins to disintegrate in about two weeks. This type, characterized by its requirement of polysaccharides for vigorous growth, as well as its morphology, is possibly related to the Cellvibrio described by Winogradsky.

Type XI also attacks cellulose, but exhibits no yellow pigment and does not digest starch. These straight motile rods were isolated from decaying crayfish and May-fly nymph shells. They do not correspond to any Cellulomonas listed by Bergey.

Type XII appears to be a well defined species, exhibiting striking morphological and cultural peculiarities. It was found in the intestine of a frog, in a sample of Bryozoa from Lake Alexander, and in mud at the waters' edge of Lake of the Isles. It is distinctly pleomorphic (figs. 8 and 9). Stained smears show rods with pointed ends, sometimes bulging to spindle shapes, sometimes elongating into gracefully curved forms. When an individual divides, it seems to pull apart rather than to cut or break sharply in two; thus, one often sees pairs joined end to end by a long thread-like structure. After a few days, pale-staining oval forms also appear. Young cultures of the frog strains are monotrichic, the others appear to be non-motile. A most striking

color effect occurs in media. A brilliant daffodil-yellow insoluble pigment is produced and in addition a brownish black soluble material which diffuses into the medium, causing the so-called "tyrosinase reaction" often seen in cultures of actinomycetes. As a result the colonies in chitin agar are a yellowish-green, and in the enrichment medium there is a thick layer of chartreuse-green slime at the surface. Chitin destruction is prompt. Thus far, no strains have visibly destroyed the paper in cellulose tubes, though they all produce a yellow growth upon it very promptly. Eight other motile strains, culturally and morphologically similar, but lacking pigmentation, should probably be regarded as variants. Three of them blackened the chitin cultures after two or three days.

Type XIII also is conspicuous in color. It was isolated from the stomach of a bat, from crayfish, from the mud at the edge of a stagnant pool, and from sand under running water. In the enrichment medium the chitin strip is colored a clear daffodil-yellow beneath the surface of the liquid. The portion exposed to the air takes on a rich orange shade. Disintegration is delayed, but ultimately takes place. Stained smears from these tubes show small monotrichic rods (fig. 10), but those from agar slants exhibit the fusiform and spindle shapes seen in type XII (figs. 11 and 12). On agar media the growth is yellow. Pellicles form on liquids. Cellulose is not visibly destroyed, though it supports a prompt yellow growth.

A few gram-negative pigmented forms remain to be discussed. Two brilliantly colored strains of Pseudomonas were isolated, one from the stomach of a bat, the other from a crayfish. Pigmentation was typical on all the media. The chitin destruction was rather slow, but these cultures probably merit designation as type XIV.

Four strains from Lake Alexander, characterized by a pinkish color on the chitin strips, but nowhere else, make up type XV. The growth on potato is yellow to orange rather than pink; no pigment appears on any other medium.

Sixteen strains which exhibit consistent yellow or orange pigmentation on all media have not as yet been designated as

special types, for several reasons. Some of them occasionally show morphological similarities to types XII or XIII, others, culturally very similar, never do. Moreover, their power to attack chitin has been lost on successive transfers in most cases.

A striking feature of this investigation is the almost complete absence of gram-positive forms. In the original mixed cultures they are often present, but only two were found capable of attacking chitin when in pure culture. As they are dissimilar in many respects they must be designated as separate types.

Type XVI, then, consists of a single culture isolated from the bottom mud of Lake Alexander. It may belong to the genus Bacillus, for the motile rods are square-ended, and tend to form in chains, and its abundant growth on the surface of media shows that it is probably not a Lactobacillus. No spores have been observed, but no special methods have been used to provoke their formation.

Type XVII, isolated from a crayfish, is a totally different kind of organism. Though it is motile in hanging drops, stained smears show the pleomorphic forms characteristic of the Corynebacteria. It produces a heavy surface growth on liquids, and on solid media a thick opaque, dull growth, which gradually takes on an eggshell pink color.

DISCUSSION

The description given by Benecke is so meager that it is impossible to tell whether any of these cultures belong to the same species as his *B. chitinovorus*. He states that gas was neither abundant nor completely absent in his chitin cultures, but makes no note as to its occurrence in sugar broth tubes. The assurances of Folpmers and Rammelberg that their organisms are identical with his, are apparently based on absence of recorded differences rather than upon convincingly identical positive findings.

No attempt has been made to group the Minnesota types into genera or to name them. It seems reasonably certain that they are too dissimilar, both in morphology and in biochemical activities, to belong to any one genus. The curved and spindle-shaped

pleomorphic forms must be species as unrelated to the simple rod-shaped chitinovores as Cellvibrio and Cytophaga are to Cellulomonas. If the cultures here reported are by any chance a fair sample of the chitinovorous bacteria in nature, all that could be said about the group as a whole is that most of them are gram-negative and monotrichic. To even this simple generalization at least five of the types described would be exceptions. Of all the biochemical properties that can be determined, the nature of the desmolases secreted by an organism, and consequently its type of fermentative action upon available sugars, is perhaps the most fundamental. The general experience seems to be that it is more significant than the particular sugars which an organism can attack. It is gratifying to note that the classification on this basis is entirely compatible with the morphological findings, and in general with the other biochemical characteristics. It is hoped that the data here assembled are sufficient to define the types so that cultures isolated in the future can easily be recognized as belonging to one of them or to a new and different type.

SUMMARY

1. About 250 pure cultures of chitinovorous bacteria have been isolated from a variety of sources, and classified into seventeen types.

2. Most of these types have not previously been described.

3. The types are so widely dissimilar both in morphology and physiology that they obviously cannot belong to any one genus.

4. Some of these types might be introduced as new species into genera now recognized. Others might be combined with some of the cellulose bacteria described by Winogradsky into a new genus characterized by their peculiar morphology and their ability to break down polysaccharides.

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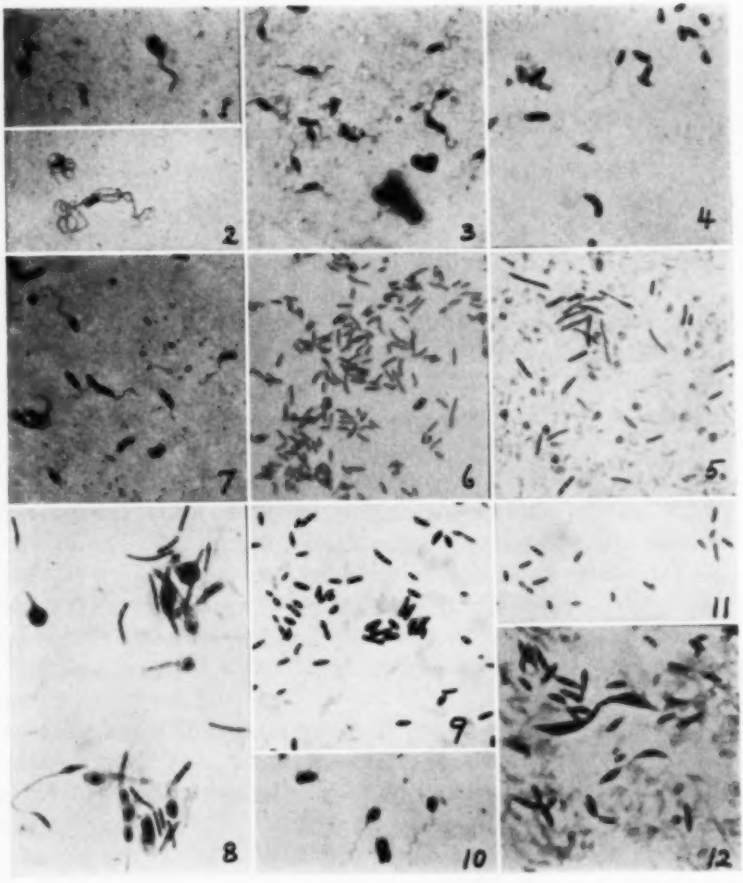
PLATE 1

(Photomicrographs × 2025)

FIG. 1. Type I, flagella stain, young culture on chitin medium
FIG. 2. Type III, flagella stain, young culture on chitin medium
FIG. 3. Type VI, flagella stain, young culture on chitin medium
FIG. 4. Type IX, flagella stain, young culture on chitin medium
FIG. 5. Type IX, Gram stain, twenty-four-hour agar slant culture
FIG. 6. Type X, Gram stain, young agar slant culture
FIG. 7. Type X, flagella stain, young culture on chitin medium
FIG. 8. Type XII, Gram stain, agar slant culture about four days old
FIG. 9. Type XII, Gram stain, agar slant culture twenty-four hours old
FIG. 10. Type XII, flagella stain, young culture on chitin medium
FIG. 11. Type XIII, Gram stain, seventy-four-hour culture on chitin medium
FIG. 12. Type XIII, Gram stain, seventy-four-hour culture on agar slant

Note. The last two were inoculated at the same time from the same inoculum, and incubated together.



(Anne G. Benton: Chitinovorous Bacteria)

STUDIES ON SMALL COLONY VARIANTS OF STAPHYLOCOCCUS AUREUS

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Small colony variants, or G forms, of bacteria have attracted considerable attention among students of bacterial variation as a result of Hadley's work (1931). In addition to a description of these variants and of the conditions under which they could be obtained, he also advanced evidence of their filterability with the belief that they represented a stage in the life cycle of the organism.

While Hadley's publication has provided the stimulus for recent investigation of these forms, the occurrence of what were in all probability similar variants had been noted by others. Since this work has been reviewed by Edwards and by Koser and Dienst, it is unnecessary to detail it here. It is believed, however, that the outline presented in Table I may be useful as a summary of the occurrence of these forms as they have been observed to date.

In the present investigation small colony variants of *Staphylococcus aureus* were studied with respect to their occurrence, morphology, physiological and antigenic properties, and filterability.

The usual type of variation encountered among the staphylococci has been a change in chromogenesis, the derivation of "albus" variants from "aureus" strains. Anyone who has worked with cultures of *Staph. aureus* has undoubtedly observed this change frequently. Pinner and Voldrich (1932) compared such

[1] The writer is indebted to Dr. S. A. Koser for suggestions and helpful criticism in the preparation of this manuscript.

"albus" as well as "roseus" and "citreus" variant forms with the original strains from which they had arisen and found them to differ in biochemical properties and in virulence, the variants being relatively avirulent. They did not observe the occurrence of rough colonies nor of any tiny colonies in the strains they studied. Bigger, Boland and O'Meara (1927) described six variant strains of *Staph. aureus* which differed in three properties, namely color, texture (smoothness or roughness of the colony) and viscidity. Various types of rough colonies and a small colony or G form were obtained from a single strain of *Staph. aureus* by Hoffstadt and Youmans (1932). This G form was isolated from lithium chloride medium and from rabbits inoculated with a smooth strain of the staphylococcus. More recently the same authors (1934) have described several white variants and G forms from orange strains of staphylococci. The G forms described by these authors illustrate well the fact that small colony forms are not all alike, for they differ in many ways from the small colony strains described in this paper.

ORIGIN OF THE SMALL COLONY STRAINS

The first small colony strains to be isolated by us were obtained from an agar slant culture of *Staph. aureus* which had been kept in the ice-box for three months and subsequently at room temperature for three weeks. Examination of plates made from this culture revealed several minute, colorless colonies scattered among the larger orange colonies typical of *Staph. aureus*.

Experiments with varied environmental conditions made in order to determine factors which favored formation of small colony variants included aging on agar and in broth, daily transfers and aging cultures in lithium chloride broth, and aging in 0.5 per cent casein digest medium. Plates were streaked from these cultures at twenty-four and forty-eight hours and weekly for a period of four weeks.

So far as could be observed, the age of the culture is especially associated with the formation of small colony variants, although other influences may play a part. Tiny colonies were not obtained from very young cultures, except occasionally from broth contain-

TABLE 1

Occurrence of small colony variants

DATE	INVESTIGATOR	ORGANISMS	CONDITIONS
1910	Jacobsen	*Eberthella typhosa*	Blood culture on Conradi agar autoclaved 30 minutes. Normal on agar autoclaved 15 minutes
1911	Fromme	*Eberthella typhosa*	Typical when isolated. Small colonies on subsequent transfer on agar
1914	Eisenberg	*Eberthella typhosa*	Old blood-broth and blood-bile cultures
		Serratia marcescens	Peptone water (7 per cent) cultures. Cultures on media containing various dyes
1918	Baerthlein	*Eberthella typhosa*	Typhoid stools streaked on Conradi-Drigalski medium
		Vibrio cholerae	Cholera stools on hemoglobin-extract-alkali-soda-agar
1922	Fürth	*Salmonella aertrycke* *Salmonella Schottmülleri*	Old broth cultures
1930	Koser	*Salmonella aertrycke*	Old broth cultures
1931	Edwards	*Shigella equirulis*	Direct cultivation, 25 per cent post-mortem examinations. Also in typical stock cultures
1931	Hadley	*Shigella dysenteriae* and other cultures	Serial transfers in $LiCl_2$ broth. Serial transfers in pancreatin broth. Aging nutrient broth cultures. Bacteriophage filtrates by "serial plates"

TABLE 1—*Concluded*

DATE	INVESTIGATOR	ORGANISMS	CONDITIONS
1931	Kuhn and Sternberg	*Escherichia coli* (45 strains) *Vibrio Metchnikovii* *Salmonella aertrycke* or *S. enteritidis* (mouse typhoid) *Salmonella suipestifer* *Pasteurella suiseptica* *Corynebacterium diphtheriae* *Proteus vulgaris* Proteus X19 *Shigella dysenteriae* *Eberthella typhosa* *Spirillum volutans* *Bacillus anthracis* *Bacillus sp.* (pseudoanthrax) *Mycobacterium tuberculosis*	Nutrient broth containing ammonia. Brief exposure to dilute phenol
1932	Hoffstadt and Youmans	*Staphylococcus aureus*	Serial transfers in $LiCl_2$ broth. Intravenous inoculation of rabbits
1933	Novak and Henrici	Pleomorphic organism between staphylococci and actinomycetes	Old broth cultures (7 weeks)
1933	Rettger and Gillespie	*Bacillus megatherium*	Serial transfers in $LiCl_2$ broth at 22°C.
1934	Koser and Dienst	*Shigella paradysenteriae* var. Sonne	Aging casein digest cultures. Old agar slants
1934	Raney and Kopeloff	*Lactobacillus acidophilus*	Old broth cultures
1934	Roe	*Clostridium Welchii*	Aging colonies and broth cultures
1934	Roos, Reichel and Clark	Hemolytic streptococcus	Brain broth containing 5 per cent normal rabbit blood

ing lithium chloride. They appeared after the cultures had aged for a week or two and were most numerous after three or four weeks. Lithium chloride had little effect in stimulating this

type of variation. Continuous transfer failed to bring out any large number of the small colony forms, and the few that appeared occurred irregularly.

In the cultures studied tiny colonies never occurred in large numbers, the most numerous being in the ratio of one small to sixteen large colonies. Usually there was only one small colony to 100 or more large colonies, and many times no minute colonies appeared. These small colony variants were unstable for the most part. Often the small colonies originally seen reverted wholly to the normal[2] form after two or three transfers on agar plates. Of several hundred colonies isolated, only nine remained small on repeated subculture over a period of several months. These nine were studied in the experiments reported in this paper.

A brief description of the origin of each variant strain follows.

V1, V2, V3: From an agar slant culture of *Staph. aureus*, strain M23, which was isolated originally from a furuncle. The slant had been incubated for 48 hours at 37°C. and then stored in the ice box for approximately three months. It had then been kept at room temperature for three weeks. The culture was rather dry and somewhat translucent.

V4, V5: From an agar slant of *Staph. aureus*, strain 95, which had been obtained from a case of food poisoning. The culture had been kept at room temperature for three weeks, at which time several small colonies appeared on plates streaked from it.

V6: From the third tube in a series of daily transfers of the Norsenski strain of *Staph. aureus* in 0.25 per cent lithium chloride broth.

V7: From the seventh tube in the same series as the preceding.

V8: From a culture of strain A of *Staph. aureus* which had been aging in 0.25 per cent lithium chloride broth at room temperature for one month. Strain A had been isolated from a case of osteomyelitis.

V9: From a nutrient broth culture of strain A which had been at room temperature for one month.

V10: From an agar slant of *Staph. albus* which had been kept at room temperature for two months. The strain reverted to the normal form on the second transfer so that no further studies were carried out with it.

[2] The word "normal" is used in this paper as the most convenient term to designate the type of colony of *Staph. aureus* usually observed, which is round and smooth, with a diameter of 1 to 2 mm. when grown on nutrient agar. recognized that this may not necessarily be the only normal form.

V11: From a culture of strain 105 in 0.5 per cent casein digest broth, kept at 37°C. for three weeks. All of the strains isolated returned to normal after two or three transfers. Strain 105 was originally isolated from a case of food poisoning.

MORPHOLOGY AND COLONY FORM

Well-isolated colonies of *Staph. aureus* are usually from 1 to 2 mm. in diameter when grown on nutrient agar. Those of the small colony variants could scarcely be discerned without the aid of a microscope. In the various strains they ranged from 0.04 mm., the smallest ever observed, to 0.5 mm., but most frequently they were 0.1 mm. in diameter. The limits of variation were quite constant for each strain, except when the culture appeared to be returning to the normal, at which time all sizes of colonies occurred.

With the exception of one strain, colonies of the variants were all smooth, round and convex, with entire margins. Strain V1, however, usually produced flat, rough colonies with slightly irregular margins. These variants had lost the power of pigment production.

Morphologically the variants were not strikingly different from the original strains, cells of which were about 0.8 micron in diameter, gram-positive, and arranged in irregular clusters. The cells of the different variants showed some individual differences and will be described separately. Several were similar to the normal form, gram-positive and grouped in small clusters or pairs. They were perhaps a trifle smaller than the usual form. Strain V3 showed a tendency to be gram-negative.

In variants V1 and V2 the cells were very irregular in size. Some were in tetrads, others in pairs or single. Within each group of cells there were marked contrasts in size. A tetrad might consist of one very large, balloon-like cell and three small ones, or two large and two small ones. When large cells occurred they were usually oval, about 1.8 by 0.9 microns, while the small ones were 0.5 micron in diameter.

Strains V4 and V5 also showed some variation in morphological characteristics. The organisms were in tetrads or pairs

and were not always spherical. The larger ones were about 1 by 1.2 microns and the smaller about 0.8 micron.

REVERSION OF VARIANT TO NORMAL FORM

Aging in nutrient broth. In order to study the return of the small colony to the normal form, the strains were inoculated into nutrient broth of pH 7.0, and incubated at 37°C. The development of these cultures was followed by streaking agar plates at intervals and observing the appearance of the colonies after forty-eight hours' incubation.

In the first series most of the strains were found to have a long lag phase. All of the tubes except one failed to show clouding at the end of twenty-four hours, although plates streaked at this time with a 1 mm. loop showed from 1 to 50 colonies. In forty-eight hours there was still no turbidity in any of the cultures. Only one culture, V1, showed clouding at the start and numerous colonies each time it was streaked on a plate. At the end of six days there was some clouding in all the tubes, and in some strains colonies of normal size (1 to 2 mm.) appeared along with the small colonies on plates.

Considerable difference was shown between the strains in their behavior in broth and in the rate at which they returned to the normal. These differences were apparent even in sub-strains which came originally from the same colony, notably V3a and V3b. V3a produced very minute colonies, 0.07 to 0.14 mm., at the end of forty-eight hours. After six days they were about 0.3 mm., and 60 per cent were 1 mm. in diameter after two weeks. After four weeks' aging in broth the colonies were all large. Strain V3b, on the other hand, never gave rise to a colony larger than 0.25 mm. In contrast to these is the behavior of strain V6 which showed 50 per cent normal colonies on plates streaked from twenty-four-hour broth cultures.

Spontaneous appearance of normal form. Reversion to the normal frequently occurred spontaneously. Sometimes in transferring from a stock culture, one or two large colonies with deep golden pigment appeared on the new slant and stood out sharply against the background of very small colonies which were scarcely

visible. Also in streaking plates from stock cultures of variants several large orange colonies occasionally appeared scattered among the more numerous tiny ones. This sudden reversion from variant to normal is quite different from the very gradual change described by Hadley for the G form of the Shiga dysentery bacillus and by Koser and Dienst for the Sonne dysentery bacillus. It is apparently due to changes taking place in an occasional cell but not affecting the culture as a whole.

Effect of glucose. This sugar had a marked effect on reversion as several strains showed almost 100 per cent normal colonies after forty-eight hours in glucose broth. All of the variants were streaked on plates of nutrient agar, veal-infusion agar, nutrient agar containing 0.5 per cent glucose, and veal-infusion agar to which 0.5 per cent glucose had been added, incubated under the same conditions and examined after forty-eight hours' growth.

Differences in behavior on these media were quite apparent. The colonies on nutrient agar were very small and only two strains formed any normal colonies. On veal-infusion agar the colonies were all larger, and two strains showed numerous normal colonies. Glucose added to nutrient agar also seemed to favor growth, as the colonies on this medium were distinctly larger than those on agar without glucose. The combination of glucose and veal infusion had the most marked effect. All of the strains which had ever reverted to the normal formed at least a few normal colonies on glucose veal-infusion agar. To illustrate these differences, the average colony measurements of one strain on the various media are given. The colonies of V2 were exceedingly small, 0.04 to 0.07 mm., on nutrient agar. On veal-infusion agar they ranged from 0.07 to 0.2 mm. On glucose agar they were 0.25 to 0.5 mm., with a few tiny colonies scattered on the plate, but on glucose veal-infusion agar they all looked like normal staphylococcus colonies, 1.0 to 1.8 mm. and of a deep orange color. Such studies indicate that the production of these small colony variants was linked with some form of altered metabolism since a change in the medium had such a profound effect on the variants.

Failure to obtain reversion. Three strains resisted all attempts

to make them produce normal colonies. The colonies of strain V1, which were small, flat and rough, never attained a size of more than 0.5 mm. in diameter. In addition to aging in broth, serial transfers were carried out in broth and on agar plates, the largest colonies being selected for transfer. Only small flat colonies continued to develop. Colonies on blood agar were even smaller than on nutrient agar. Strains V4 and V5 also failed to return to the normal. They showed no change after repeated transfers in broth and on agar slants. On blood agar, however, they developed larger orange colonies quite uniform in size and at least 1.0 mm. in diameter. These colonies did not represent a permanent return of the variant to the normal, for when transferred from blood agar to nutrient agar only very tiny colonies appeared. Since these strains failed to return to the normal, there is the possibility that they were contaminants rather than variants of the original staphylococcus. However, since their cultural and morphological characteristics on blood agar resembled so closely those of the original cultures, it is believed that they really represent variants which did not return to the normal on a standard medium within the period of observation.

In this group of small colony strains the cultures exhibited all degrees of stability, ranging from those which continually gave a few large colonies on each subculture to those which never reverted wholly to the normal.

INFLUENCE OF MEDIUM UPON GROWTH

Effect of blood. In addition to complete reversion to the normal a temporary stimulation of growth was found to occur under certain conditions. After twenty-four hours' growth on blood agar the colonies were all small, appearing much as they did on nutrient agar. At the end of forty-eight hours the colonies of two strains had become 1 mm. in diameter and had acquired a pale orange color. They looked like typical staphylococcus colonies, but differed from those of the parent strain in that the colonies were smaller and not hemolytic. When transferred back to nutrient agar only tiny colonies developed. Although cultured repeatedly on blood agar, they did not become hemolytic nor did

they acquire permanently the property of forming large colonies when cultured again on nutrient agar. None of the other variants showed this sudden development of all the colonies to a large size.

Effect of other organisms. In examining some old nutrient agar plates of strain V4 upon which two contaminants had appeared, it was observed that around the two stray colonies the colonies of the variant had become quite large and slightly pigmented. This appearance was confined to the immediate vicinity of the contaminating colony. The effect was similar to the "satellite phenomenon" shown by mixed cultures of *Staph. aureus* and *Hemophilus influenzae* on blood agar in which the *H. influenzae* grows much more luxuriantly in the neighborhood of a staphylococcus colony. The contaminants were large, non-motile, gram-positive, spore-forming rods.

These two organisms and also a laboratory stock culture of *Bacillus subtilis* were studied for their effect upon all of the small colony variants. Nutrient agar plates were streaked with cultures of the variants and a single streak of the other organism was made along the center of the plate at right angles to the other streaks. The plates were examined at the end of forty-eight hours at 37°C. and again at the end of ten days at room temperature. *B. subtilis* had no effect upon any of the small colony strains. The two contaminants affected two variants very markedly. Strains V4 and V5 grew much more luxuriantly near the center of the plate where the contaminant was streaked than at the edge of the plate. There was a gradual gradation in size from the larger colonies adjacent to the contaminant to those only slightly enlarged about 1 cm. away. Beyond that the colonies were uniformly small. The nature of the growth-promoting substance produced by these contaminants is unknown.

Effect of glucose and veal infusion. The influence of glucose in causing reversion of the variants to the normal form has been noted. It had also been observed in other experiments that colonies were sometimes larger on veal infusion than on nutrient agar, especially in the case of strains V4 and V5 whose growth has already been shown to be particularly subject to changes in the

composition of the medium. Strain V5 formed only very tiny colonies, 0.07 mm. to 0.13 mm., on nutrient agar. On veal infusion the colonies were 0.5 mm. to 0.6 mm., on glucose agar they were all about 0.2 mm., and on glucose veal-infusion agar they were 1.0 mm. across. However, these large colonies formed only tiny colonies when transferred again to nutrient agar.

FERMENTATION REACTIONS

The fermentation reactions of the small colony variants were compared with those of the original strains from which they were obtained and also with cultures which had been brought back from the small colony state to normal colony size by aging in broth or by other methods.

The cultures were inoculated into broth tubes containing 0.5 per cent of glucose, lactose, or sucrose, with brom thymol blue as an indicator. The reactions, as indicated by changes in the indicator, were observed at the end of twenty-four hours, forty-eight hours, one week, and two weeks, at which time plates were streaked.

The original staphylococcus strains, with one exception, attacked glucose, lactose, and sucrose, and produced a definite acid reaction in twenty-four hours. The variants differed in the time required to show any reaction and in the sugars which they were able to utilize. After twenty-four hours some of the tubes showed very slight clouding but there was no change in the indicator. At the end of forty-eight hours all of the glucose tubes showed abundant growth and a distinct acid reaction. Plates streaked from these cultures at times yielded only small colonies, while at other times both small and large colonies appeared. Four strains produced acid and formed only tiny colonies. One strain formed acid from glucose and the plates yielded mostly tiny colonies with a very few normal ones, while another fermented glucose and showed normal and small colonies in about equal numbers. When some reversion from the variant to normal had occurred by the time acid production was first evident, it was difficult to decide whether the carbohydrate had been attacked by the variant, by the normal form, or by both. How-

ever, since fermentation of glucose did occur in some tubes in which there was no reversion, or only slight reversion, of the culture to the normal, it may be presumed that the small colony variants did attack glucose, although somewhat more slowly than the normal strains.

Lactose not only was not fermented by the small colony strains but seemed to be inhibitory to their growth. Most of the tubes showed no clouding at the end of twenty-four hours' incubation and few or no colonies appeared on plates streaked from them. After forty-eight hours some of the tubes still showed no clouding and only scant growth on streaked plates. In a week there was moderate clouding in most of the tubes, but a few strains failed to grow. The acidity of the medium never became greater than pH 6.8.

The reactions in sucrose were similar to those in lactose. There was usually no clouding or change in reaction in twenty-four hours and only slight clouding in forty-eight hours. Two strains, V5 and V6, produced an acidity of pH 6.3 at the end of a week. The former had reverted almost completely to the normal at the time that this acidity was first observed.

The following table (Table 2) presents some of the reactions produced by these variants in glucose, lactose, and sucrose broths. It illustrates the feeble fermentative capacity of the small colony variants. In contrast, all of the strains which had been brought back to the normal fermented glucose, lactose and sucrose as readily as did the original normal strains.

BEHAVIOR OF VARIANTS UNDER ANAEROBIC CONDITIONS

In order to compare the growth of both the variant and normal strains under aerobic and anaerobic conditions, nutrient agar plates were streaked in duplicate from each culture. One set of plates was placed in a vacuum desiccator, evacuated until the pressure in the jar was reduced by 700 mm. of mercury and any remaining oxygen absorbed with pyrogallic acid and sodium carbonate. The other set of plates was kept under aerobic conditions. Both aerobic and anaerobic plates were incubated at 37°C. for forty-eight hours.

The normal cultures grew luxuriantly and produced abundant

pigment in the presence of oxygen, while under anaerobic conditions the colonies were slightly smaller in size and showed no

TABLE 2

Fermentation reactions of small colony variants

STRAIN	CLOUDING	REACTION	COLONIES
		Glucose*	
V1	Moderate	6.5	Many, all small
V2	Abundant	6.3	50 per cent normal; 50 per cent small
V3a	Moderate	6.5	Few, all small
V3b	Abundant	6.3	Many, all small
V5	Abundant	6.3	Many, all small
V6	Abundant	6.0	33 per cent normal; 67 per cent small
V7	Abundant	6.0	33 per cent normal; 67 per cent small
V8	Abundant	6.0	Many small; few 0.2 mm.
V9	Slight	6.7	Few, small
		Lactose	
V1	Abundant	6.8	Many, small
V2	None	6.9	None
V3a	None	6.9	Very few, small
V3b	None	6.9	None
V5	Moderate	6.9	Many, all small
V6	Moderate	6.9	98 per cent normal; 2 per cent small
V7	Moderate	6.9	98 per cent normal; 2 per cent small
V8	None	6.9	None
V9	None	6.9	Many, all small
		Sucrose	
V1	None	7.1	None
V2	None	7.1	None
V3a	None	7.1	2 colonies, small
V3b	None	7.1	None
V5	Moderate	6.9	Few, all small
V6	Abundant	6.9	98 per cent normal; 2 per cent small
V7	None	7.1	None
V8	None	7.1	None
V9	None	7.1	None

* These are the reactions after 48 hours' incubation. The reactions in lactose and sucrose showed no change after 7 days' incubation.

pigment. Most of the variants grew better aerobically than anaerobically as indicated by the number and size of the colonies.

Usually there were distinctly fewer colonies on the anaerobic than on the aerobic plates. The colonies which developed in the absence of oxygen were often extremely minute. Thus the aerobic colonies of strain V5 were about 0.1 to 0.13 mm., while the anaerobic colonies were less than 0.01 mm. in diameter. Anaerobic conditions seemed to inhibit reversion to the normal form in those strains which had a tendency to revert under the usual aerobic conditions.

FILTRATION EXPERIMENTS

The question of filterability of small colony variants is of especial interest in view of the controversy as to whether bacteria possess a filter-passing stage in their life history. Although the morphology of these small colony variants of staphylococci would suggest the impossibility of their passage through Berkefeld filters, small units representing a filter passing stage might be associated with the large cells. Tests for filterability were made with both normal and variant strains.

Preparation of cultures. Kendall's K medium (1931) was used in the first series of filtrations. Cultures were inoculated into 150 cc. amounts of the medium and samples were withdrawn for filtration after forty-eight hours and at weekly intervals for one month. Cultures were also subjected to daily transplants in the K medium and filtered at the end of the third, seventh and twelfth transfers. Plates were streaked from each culture before filtration in order to determine whether any visible changes in the colony form had occurred.

Cultures in nutrient broth of pH 7.4 were also used. Samples were withdrawn for filtration at the end of forty-eight hours, one week and four weeks. Plates were streaked at the time of each filtration in order to determine the proportion of cells in the small colony state. Various degrees of reversion were revealed by these plates from only tiny colonies at the end of forty-eight hours to 90 per cent normal colonies after a month.

Technique of filtration. Berkefeld N filters shown to be impervious to a twenty-four-hour-broth culture of *Serratia marcescens*

were used. Usually about 15 cc. of the culture was filtered. The time required for the filtration was short, usually about five minutes. The filtrates were collected in test tubes within the suction flasks.

Tests of filtrates. Each filtrate in amounts of 1.0 cc. and 0.25 cc. was inoculated into 10 cc. tubes of glucose veal-infusion broth. K medium was used, in addition, for all of the K medium filtrates. The broth tubes were held at 37°C. for one month and examined at intervals for the appearance of any sign of growth. The Hauduroy technique of serial plate transfers was also used. Material was transferred from plate to plate at intervals of forty-eight hours for 6 transfers, the plates being examined microscopically each time before a fresh transfer was made. The remainder of each filtrate was kept at room temperature for one month, examined for growth and a few drops spread on an agar plate.

Results of filtration tests. Although a total of thirty filtrations was made, growth could not be detected in any of the media inoculated with filtrates from variant or from normal cultures. The broth tubes and the tubes of filtrate allowed to stand at room temperature occasionally developed a slight cloudiness, but stains made from these tubes showed nothing except a slight precipitate or bits of débris. Plates heavily inoculated with material from these cloudy tubes showed no growth.

As the dried broth accumulated on the plates used for the Hauduroy transfer technic after about the third or fourth transfer it formed a thin film, but stains made of this film showed nothing that might be interpreted as being a bacterial cell. In subsequent transfers discrete colonies could not be detected in the place of the film.

PATHOGENICITY

During the preparation of agglutinating sera it was observed that rabbits receiving intravenous inoculations of certain of the normal strains frequently became emaciated and sometimes died, while animals which received the variants did not develop unto-

ward symptoms. Such evidence suggested a loss of virulence of the small colony strains. The following experiments were carried out in order to verify this point.

Two series of cultures were selected. The first comprised normal strain A, a variant of this strain, V8, and a normal strain, V8-N, recovered from the variant; the second, strain 95 and its variant, V5. The organisms were grown on agar slants for twenty-four hours, suspended in saline, and standardized. Young rabbits weighing about 1500 grams each received intravenously one billion organisms of the strain under test. The animals were

TABLE 3

Effect of staphylococci upon rabbits

STRAIN	TYPE	LOSS IN WEIGHT	GROSS PATHOLOGY	SMEAR OF PUS FROM KIDNEY LESION
		grams		
A	Normal	300	Multiple abscesses in both kidneys	Gram-positive cocci
V8 (from A)	Small colony	None	None	No organisms
V8—N	Normal from variant	None	Pale area with tiny abscesses in one kidney	Gram-positive cocci
95	Normal	125	None	No organisms
V5 (from 95)	Small colony	Gain	None	No organisms

watched and changes in weight recorded for a week, after which they were killed and examined for gross lesions. Table 3 summarizes the changes observed.

The results with strain 95 and its variant are not conclusive because the normal organisms lacked sufficient virulence to produce macroscopic lesions in the rabbit although a loss of weight was observed. With strain A the loss in weight following injection, together with the renal abscesses seen at autopsy indicate a certain degree of virulence. In contrast to this is the complete lack of any detectable change in the rabbit which received an equal number of organisms of the variant strain. These results confirmed the previous observations that these variants are relatively avirulent. The behavior of the organism which had

regained its normal form from the variant condition was inconclusive. It did give rise to a few lesions in one kidney, but not to the extent observed with the original strain.

This decrease in virulence would lead one to believe that these particular variants are of slight importance in disease processes.

AGGLUTINATION

The original strains were not serologically specific, all showing some cross agglutination. Variants of 3 of the 4 normal strains studied were antigenically similar to their source strains as determined by agglutination reactions, and the same variants showed a like antigenic relationship to each other. The titers of the sera prepared against the parent strains were somewhat lower when tested with their variants. In the fourth instance the two variants from strain 95 showed no agglutination with 95 serum. These organisms behaved in a somewhat anomalous manner in other reactions and had never reverted to any permanent normal forms.

Sera prepared against the small colony strains likewise agglutinated the normal organisms, although the titers were rather low against both the variants and the normal bacteria. This was probably due to the fact that only killed bacteria were used in the process of immunization to prevent the possibility of any reversion to the normal taking place after inoculation. The following table illustrates the results obtained in agglutination tests.

The findings are at variance with the report of Hoffstadt, Youmans, and Clark (1934) that the G organisms of *Staph. aureus* were agglutinated only by G sera. They support the idea that this type of variation represents a change in physiological behavior rather than alteration in the actual composition of the organisms.

RATE OF MULTIPLICATION

The behavior of the small colony variants under the conditions in which they were studied pointed either to a very prolonged lag phase, a much retarded growth rate, or to a combination of both of these factors. A more accurate comparison of the rates of multiplication of normal and variant staphylococci was obtained

by following quantitatively the increase in the numbers of cells in broth cultures of the organisms. The normal strain, A, and variant, V8, were selected because V8 had proved to be quite stable, so that the results would not be confused by the reversion of numerous variant cells to normal. For further comparison a strain, V8-N, which had reverted from the variant to the normal form, was also used.

The organisms were grown on agar slants for twenty-four hour and suspensions in saline of approximately equal turbidity were

TABLE 4

Agglutination of various staphylococci with 95 (normal) serum

STRAIN	CONDITION	1:40	1:80	1:160	1:320	1:640	CONTROL
95	Normal	4+	4+	4+	3+	2+	–
V4	Variant from 95	–	–	–	–	–	–
V5	Variant from 95	–	–	–	–	–	–
Norsenski	Normal	4+	4+	2+	1+	1+	–
V7	Variant from Norsenski	2+	1+	1+	–	–	–
M23	Normal	4+	4+	4+	4+	3+	–
V1	Variant from M23	3+	3+	3+	3+	2+	–
V2	Variant from M23	4+	4+	3+	3+	3+	–
V3	Variant from M23	3+	2+	1+	–	–	–
A	Normal	4+	4+	4+	3+	2+	–
V8	Variant from A	4+	4+	4+	3+	2+	–

prepared. One 1 mm. loop of each suspension was inoculated into 100 cc. of broth. Plates were poured immediately to determine the number of viable organisms per cubic centimeter in each culture at the beginning of the experiment. Appropriate dilutions in sterile saline were made and plates poured in duplicate at the end of two, four, eight, twelve, twenty-four, and forty-eight hours. These plates were incubated for three days at 37°C. to insure the development of all colonies, especially the tiny ones, to a visible point. The numbers of colonies were counted and the numbers of viable organisms per cubic centimeter in the culture at each time of plating were estimated. From these figures the

progressive increase in the number of cells in the cultures could be calculated. Table 5 is typical of the results obtained.

The contrast between the rate of multiplication of the small colony variant and that of the normal strain is brought out sharply by the figures in the second part of the table which express approximately the ratio of the number of organisms to the original number at each time of plating. For the first two hours,

TABLE 5

Multiplication in nutrient broth

TIME	NORMAL STRAIN A	VARIANT V8	NORMAL RECOVERED FROM VARIANT V8-N
	Colonies per cubic centimeter		
hours			
0	2,850	2,080	2,120
2	3,800	4,530	3,340
4	31,300	5,870	17,300
8	8,500,000	19,600	22,900,000
12	113,000,000	33,300	79,000,000
24	370,000,000	960,000	520,000,000
48	790,000,000	113,000,000	1,010,000,000
	Ratio to original inoculation		
0	1	1	1
2	1.3	2	1.6
4	11	2.8	8.2
8	3,000	9.4	10,800
12	40,000	16	37,300
24	130,000	460	245,000
48	277,000	54,000	476,000

which probably represents the period of lag, the three cultures behaved very much alike, the increase in numbers being relatively slight. At the end of four hours, both normal strains were multiplying distinctly more rapidly than the variant. In twelve hours the number of viable cells per cubic centimeter in the variant culture was only sixteen times that of the original inoculum, while in the normal strain the number was 40,000 times that at the

beginning of the experiment. Similar differences are quite apparent in the figures for twenty-four hours and forty-eight hours. The strain V8-N, which had reverted to the normal, appeared to increase as rapidly as the original normal strain. The cultures were not followed after forty-eight hours because at this time several normal colonies appeared on plates streaked from the variant cultures. Such organisms, which presumably had recovered their more rapid rate of reproduction, would confuse further study of the slower growing variants.

DISCUSSION

All attempts to demonstrate the association of a filterable or "virus-like" stage with these organisms were unsuccessful. The results differ in this respect from those of Hoffstadt and Youmans (1932). The fact that these forms occur rarely and that staphylococcus cultures may be studied under diverse conditions without the appearance of small colony forms would cast considerable doubt upon the assumption that they constitute an essential stage in the life cycle of the organism.

The small colony variants are characterized by a change of physiological properties. The deviation from the familiar form which first brought them to the attention of investigators was a decrease in colony size. Further studies on these staphylococcus variants have revealed a rate of multiplication much slower than that in the normal form, a decrease in fermentative powers, loss of pigment production, loss of virulence, and often an increase in the size of the individual cells. These changes seem to be chiefly physiological. The fundamental variation is probably one of metabolic activity, since the use of enriched media accelerated reversion to the normal form.

It would appear, then, that as a result of age or of some chemical injury there is damage to an occasional cell which is retained through a number of successive cell generations. This does not affect equally all of the cells in a culture. In a new environment, such as is obtained by transfer to new media, an occasional cell may recover its lost characteristics. This return to the normal likewise does not affect equally all of the organisms in the culture.

The change from small to large colonies was usually abrupt and colonies of intermediate sizes seldom appeared. At no time was there a uniform increase in colony size of all the organisms in a culture. However, the proportion of such cells recovering could be increased by enrichment of the media in which they were grown.

SUMMARY

1. Several small colony variants of *Staphylococcus aureus* were isolated from old broth or agar cultures or from cultures in lithium chloride broth. Their occurrence was irregular and rare.

2. The colonies of these organisms were very small and lacked pigment. The cells were similar to those of the normal form in some cases, while in other strains there were marked irregularities in size.

3. The small colony forms were characterized by a change of physiological properties, such as loss of fermentative powers and a relatively slow rate of multiplication. Growth could be stimulated in some strains by the use of veal infusion and glucose, and in others by the presence of blood in the medium or of certain contaminants on the plates.

4. Reversion to the normal form occurred occasionally on agar. It could be enhanced by aging in broth or by enrichment of the media.

5. The variant organisms were avirulent for rabbits in the doses used.

6. The variant organisms could not be recovered from filtrates of Berkefeld N candles.

7. Agglutination tests showed most of the variants to be antigenically similar to their source strains.

8. It is suggested that these small colony forms occur as the result of injury to an occasional cell which is retained through successive cell generations.

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PLATE 1

FIG. 1. Cells of normal staphylococcus, M23, from twenty-four-hour colonies on nutrient agar.

FIG. 2. Cells of small colony variant, V2, from twenty-four-hour colonies on nutrient agar.

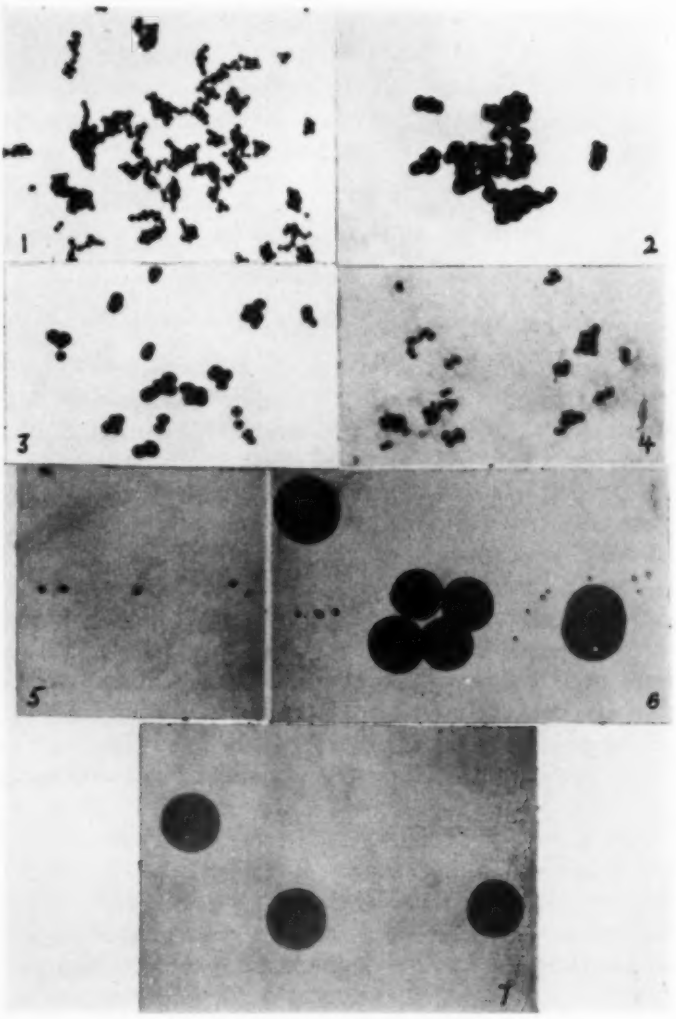
FIG. 3. Cells of small colony variant, V1, from twenty-four-hour colonies on nutrient agar.

FIG. 4. Cells of small colony variant, V5, from twenty-four-hour colonies on nutrient agar.

FIG. 5. Colonies from twenty-four-hour-broth culture of small colony variant, V2. × 10.

FIG. 6. Colonies from three-day-broth culture of V2 showing partial reversion to normal. × 10.

FIG. 7. Colonies from seven-day-broth culture of V2 showing complete reversion to normal. × 10.



(Edith L. Swingle: Variants of Staphylococcus Aureus)

AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

III. SYSTEMATIC RELATIONSHIPS OF THE GROUP

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For many years the literature dealing with the active urea-decomposing bacteria of soils and manures (reviewed by Löhnis, 1910, and Waksman, 1931) has been overburdened with the names of imperfectly defined species. A considerable proportion of the organisms appear to belong to the *Bacillus Pasteuri* group, which has been described (Gibson, 1934) as a series of ureaclastic spore-forming bacilli which either require or exhibit a preference for alkaline media containing ammonia. An attempt has been made to collect the existing type cultures of organisms which, according to their original descriptions, might be expected to be related to *B. Pasteuri*, and several strains were secured. This paper contains a brief description of the type cultures, proposals concerning the classification of the Pasteuri group, and an account of work pertaining to its differentiation from other bacteria.

INVESTIGATIONS OF TYPE CULTURES

The results of the study of type cultures and of their comparison with recently isolated strains are given in this section. In addition, a short historical account of each type is presented, in order to evaluate its claim to recognition as a species and to clarify the confused nomenclature of the organisms.

Bacillus Pasteuri (Miquel) Migula

This organism, originally described as capable of decomposing 3 grams of urea in a liter of culture per hour, has been regarded

generally as the most distinctive species of the group. The description of *Urobacillus Pasteurii* given by Miquel (1888–89; 1889–90, p. 13) is unsatisfactory according to modern standards, but the type of organism with which he dealt may be identified with reasonable confidence by its exceptional power of decomposing urea. Migula (1900) changed the name of the organism to *Bacillus Pasteuri* without amplifying its characterization. Miquel's strains appear to have been lost, but Beijerinck (1901; 1902) described an organism, *Urobacillus Pasteurii*, which he considered identical with Miquel's type. Löhnis and Kuntze (1908) provided a still more complete description, under the specific name of Migula, based on one of Beijerinck's cultures and twelve strains of their own isolation.

Three type cultures of *B. Pasteuri* were examined in this work. A strain of Beijerinck's was received from Prof. A. J. Kluyver. It is not certain that this is the strain described in Beijerinck's classical paper, but it conforms in every way to his description except in a decreased ability to form spores. Strain A1 and strain A4a, described by Löhnis and Kuntze, were received from Prof. F. Löhnis. The latter strains now appear to be incapable of forming spores, and they produce the "rougher" types of colony (Gibson, 1934, pp. 302, 306) in which the organisms frequently grow as filaments. All three strains liquefy gelatin comparatively rapidly and reduce nitrates. Beijerinck's strain is identical with a number of the most active urea-decomposing types among the recently-isolated organisms; and Löhnis and Kuntze's strains are identical with some of the variants which were produced artificially in this work. The strains received from Löhnis both require about five days at 30°C. for complete decomposition of 2 per cent urea in bouillon, indicating a considerable reduction in urease activity in the case of strain A4a since 1908.

Urobacillus Leubei Beijerinck

Beijerinck's (1901) culture, purchased from the Kral museum, proved to be a short, stout rod which forms ovoid to elliptical spores in fusiform sporangia. Its action on urea is comparatively

feeble, and it can be induced to grow on ordinary agar. The organism reduces nitrates but does not liquefy gelatin within five months. It is identical in every respect with 8 of the recently isolated strains, and it can be distinguished from *B. Freudenreichii*, as described by Löhnis (1905), only by its inability to liquefy gelatin.

Miquel (1904) used the name *Urobac. Leubei* for Leube's (1885) *Bacterium ureae*. A culture, bearing the designation *B. ureae* Leube No. 1654, was obtained from the Lister Institute. In a private communication Dr. St. John-Brooks stated that this culture was derived from a transfer of Beijerinck's *Urobac. Leubei*. It proved to be a spore former which differs in many respects from organisms of the Pasteuri group, including an inability to decompose urea. As this bacillus has been used for biochemical investigations by other workers (Birkinshaw et al., 1931) under the name *B. ureae*, it should be put on record that it shows no resemblance to its parent strain in Vienna or to Leube's (1885) *Bacterium ureae* or to Günther's (1906) *Bacillus ureae*.

Bacillus Freudenreichii (Miquel) Migula

Incomplete descriptions of *Urobacillus Freudenreichii* were made by Miquel (1889–90, pp. 367, 488) and later by Miquel and Cambier (1902). The organism was renamed *Bacillus Freudenreichii* by Migula (1900), but was properly described for the first time by Löhnis (1905) who acted on the assumption that cultures of his isolation were probably identical with Miquel's bacillus. Bierema (1909) later isolated strains of *Bacillus pumilus* and organisms intermediate between *B. pumilus* and *B. Freudenreichii*, all of which decomposed urea. This work led Löhnis (1909) to regard *B. Freudenreichii* as a variety of *B. pumilus*. It should be pointed out, however, that the bacilli which Löhnis and Bierema recognized as *B. pumilus* were probably of a type different from the Kral strain which Lawrence and Ford (1916) examined.

A culture which is reputed to be descended from one of the original strains of *B. Freudenreichii* was received from Prof. F. Löhnis, and two cultures derived from Bierema's *B. pumilus* strains from Dr. H. Glathe. All proved to be of the Mycobacte-

rium type and differed markedly from the original sporing bacilli. Similar observations on these cultures were made by Löhnis and Smith (1923). The strains designated "*B. pumilus*" differed from the "Freudenreichii" culture in several respects, including a feeble urease activity. It has been impossible to secure an authentic culture of *B. Freudenreichii* for comparative study, but it is certain that the strains described by Löhnis belong to the Pasteuri group.

Bacillus probatus Meyer et Viehoever

Viehoever (1913) gave a lengthy account of this organism based on a single culture. He also examined stock strains from other laboratories and concluded that Beijerinck's original cultures of *Urobac. Pasteurii* and *Urobac Leubei*, and one of the cultures of *B. Pasteuri* described by Löhnis and Kuntze, were identical with his organism. Having reached this conclusion, Viehoever had no justification for introducing the new name *B. probatus*, especially as he himself pointed out (p. 213) that Löhnis adequately described the urea decomposers with which he dealt. Few would consider the characterization of *B. probatus* superior or even equal to that given by Löhnis and Kuntze for *B. Pasteuri*. The name proposed by Viehoever is therefore a homonym which must be rejected. A culture of *B. probatus* was obtained from the Kral collection. The organism is a small rod which forms slightly ovoid terminal spores. It produces a comparatively slow decomposition of urea and adapts itself to ordinary media more easily than the majority of organisms in the group. Gelatin is liquefied comparatively rapidly and nitrates are reduced.

CLASSIFICATION OF THE GROUP

In the first paper of this series (Gibson, 1934) the conclusion was reached that a natural subdivision of the Pasteuri group, on the basis of the commonly used diagnostic tests, is not possible. All distinctive strains are connected by numerous transitional forms and a precise definition of species is out of the question. It was indicated, however, that the organisms may be divided roughly into three types according to the shape of spore and

urease activity, the latter being associated with conditions of growth. The three types established on these criteria each contain an extreme form; and since the organisms representing the extremes are unmistakably different, it appears to be expedient to divide the group into three species. *B. Pasteuri* and *B. Freudenreichii* fit into this classification, but a new name is required for the third species which is the most prevalent type of the group in soils. The use of urease activity as a differential criterion necessitates the selection of some arbitrary standard, and it is suggested that the complete decomposition of the urea in bouillon containing 2 per cent of this compound within forty-eight hours be taken as the characteristic of the most active organisms. The medium (Gibson, 1934) should be used soon after preparation and should be inoculated with a loopful of liquid culture, but great refinement of the test is impossible and is scarcely warranted by the behavior of the organisms.

The three species into which the Pasteuri group may be divided are differentiated as follows:

1. *Bacillus Pasteuri* (Miquel) Migula. Spores spherical or slightly ovoid and terminal or sub-terminal. All the urea in 2 per cent urea bouillon decomposed within forty-eight hours at the optimum temperature. No growth in ordinary neutral media. Best growth in alkaline (about pH 9) media containing comparatively large quantities of ammonia (about 1 per cent NH_4Cl).

2. *Bacillus Loehnisii* n. sp. Spores spherical or slightly ovoid and terminal or sub-terminal. Decomposition of 2 per cent urea in bouillon does not reach completion within forty-eight hours at optimum temperature. Generally capable of growing in ordinary neutral media. Best growth in moderately alkaline (pH 8 to 8.5) media containing ammonia (about 0.5 per cent NH_4Cl).

3. *Bacillus Freudenreichii* (Miquel) Migula. Spores ovoid to elliptical, formed in a central or excentric position. Indistinguishable from 2 in urease activity or conditions of growth.

Each of the three species contains strains which exhibit any combination of characters, other than those used in differentiating the species, within the limits of the descriptions given in the first paper of this series. There is perhaps one exception to this state-

ment: among the strains examined, those with the lowest temperature maxima (under 30°C.) all belong to *B. Loehnisii*. In the case of *B. Pasteuri* and *B. Freudenreichii*, the characterizations proposed here involve a slight modification and extension of the original descriptions and of the emended descriptions of these species given by Löhnis. Beijerinck's (1901) *Urobac. Leubei* is regarded as a synonym of *B. Freudenreichii*. The latter name is preferred, partly because it was used earlier (1889–90) by Miquel and partly because it was employed by Löhnis (1905) in the first adequate description of an organism of this type. Löhnis and Kùntze (1908) included in the species *B. Pasteuri* strains which acted slowly upon urea. There is ample justification for this attitude, but in any subdivision of the group the highly specialized organisms which correspond to the *Urobac. Pasteurii* of Miquel and Beijerinck should be differentiated from the others. *Urobac. psychrocartericus* and *Urobac. hesmogenes*, described by Rubentschik (1926), are undoubtedly strains of this group since the descriptions of these organisms contain nothing which distinguishes them from *B. Pasteuri* as defined here. The possibility of using *Urobac. psychrocartericus* to comprise strains with low temperature requirements has been considered but appears to be impracticable. Of the strains examined, those exhibiting a maximum temperature below that of Rubentschik's organism belong to the *B. Loehnisii* type and they differ among themselves in their action on gelatin and nitrates and in other features.

The classification of the Pasteuri group proposed here is recognized as distinctly artificial. It does not even possess the merit of exactness, and consequently the diagnosis of many strains will be doubtful. Other defects are the use of such criteria as urease activity, which may become reduced in pure cultures, and of spore formation, which may disappear entirely in laboratory cultures and their variants. However, the classification may be of some value from the practical standpoint and it appears to be the best that can be devised at the present time.

A discussion of the Pasteuri group would be incomplete without a reference to a number of previously described organisms, cultures of which appear to be unobtainable. *Bacillus ureae* II and III of Burri et al. (1894), *Urobacillus Duclauxii* Miquel (1889–90,

pp. 53, 122, 145) and *Urobacillus Maddoxii* Miquel (1890–91, pp. 275, 305) were imperfectly described. Although subsequent writers (Söhngen, 1909; Düggeli, 1915; Kusnetzow, 1930) have undertaken to diagnose strains of their own isolation by means of Miquel's descriptions, this could not be done with certainty, and the names of these organisms should not be used in future. It may be indicated here that the descriptions of Burri and Miquel contain nothing which is incompatible with the organisms belonging to the Pasteuri group.

Considering the tendency of many strains in this group to conceal or to lose their ability to form spores, it is not unlikely that some of the urea decomposers which have been described as non-sporers also belong to the group. This statement may apply to *Bacillus ureae* I Burri et al. (1894), *Urobacillus Jakschii* Söhngen (1909), and to *Bacterium ureae* Leube (1885), an organism which was renamed *Urobacillus Leubei* Miquel (1904) and later *Bacillus ureae* Günther (1906). All these specific names, and also others due to Miquel and Cambier, should however be discarded for the descriptions are insufficient and cultures do not appear to be available. The detailed descriptions given by Rubentschik (1926) of *Urobacterium amylovorum*, *Urobact. citrophilum*, and *Urobact. aerophilum* show that these organisms cannot be distinguished from the Pasteuri group except by inability to form spores.

The organisms carefully described by Geilinger (1917) undoubtedly belong, with the possible exception of the strain "Erde b," to the Pasteuri group. Geilinger's paper is particularly valuable for it provides a detailed account of the variations in cultural characteristics of different strains, and shows how individual organisms may embody the characters of two or more previously described species. A few of Geilinger's organisms proved to be facultative anaerobes, growing actively and decomposing urea in the complete absence of oxygen.

DEMARCATION OF THE GROUP

The descriptions given in the first paper of this series appear to differentiate the Pasteuri group from all other species of ureaclastic bacteria which have received an adequate characterization

in the past, and also from several undescribed organisms which were isolated in this work. From bacilli which do not decompose urea, the group is distinguished principally by the urease activity of its members and by the beneficial effects of alkalinity and ammonia on their growth. There are several references in the literature (especially Löhnis, 1910, and Lehmann and Neumann, 1927) which suggest that in certain bacteria the ability to bring about an active decomposition of urea may be found in some strains but not in others. The possibility should therefore be considered that organisms which have been included in the Pasteuri group in this series of papers are merely varieties of bacteria which do not hydrolyse urea. This possibility appears to be remote except in the case of those members of the group which possess feeble ureaclastic powers and the ability to grow on ordinary media. Certain strains of this type resemble *B. sphaericus* Meyer et Neide [*B. pseudotetanicus* (Kruse) Migula] as described by Neide (1904), Ford (1916) and Cunningham (1931), and other strains approach in their characters *B. tritus* Batchelor (1919). Cultures of *B. sphaericus* were obtained from Kral (1 strain), from Ford (1 strain), and from Cunningham (9 strains), and were tested in urea bouillon. Ten strains produced no action on urea, but one of Cunningham's cultures brought about a feeble decomposition. This latter organism can be distinguished from other strains of *B. sphaericus* only by its urease activity, and it is identical with two strains isolated in this work, which, on account of their slow action on urea and their ready growth on ordinary media, had been regarded as border-line types of the Pasteuri group. The existence of such organisms indicates the absence of a natural boundary, but it seems desirable for practical reasons that they should be excluded from the Pasteuri group and regarded as ureaclastic strains of *B. sphaericus*. Their growth on artificial media is not promoted by alkalinity and the presence of ammonia, and their exclusion appears to leave intact this important criterion of the Pasteuri group.

To discover possible relationships with non-ureaclastic bacteria, a considerable number of cultures isolated in this work, but found to be incapable of decomposing urea, were investigated further.

None of them is of the *B. sphaericus* type, and the great majority are differentiated from the Pasteuri group by at least one feature other than their inability to hydrolyse urea. A few of the organisms, apparently belonging to undescribed species, require alkaline media containing ammonia, and they cannot be distinguished with certainty from strains of the Pasteuri group except by their inactivity towards urea. It therefore appears necessary to fix arbitrarily the limits of the Pasteuri group so as to exclude bacilli which fail to decompose urea, and also those which do not exhibit a preference for special media, although they possess no other differential feature.

It may be questioned whether the ability to bring about an active ammonification in urea bouillon is a feature of importance from the systematic standpoint, for every grade of urease activity is found among microörganisms. The titration of urea-containing cultures of many bacteria and fungi may yield inconclusive or negative results, whereas the organisms may be shown to contain urease by the more delicate test of adding a neutral urea solution containing phenol red to a neutral emulsion of cells and incubating the mixture, along with the necessary controls, at 45°C. The active decomposition of urea is therefore an arbitrary character, but probably in no greater degree than many other criteria used in classification. For the Pasteuri group it serves at least to bring together a large assemblage of strains among which there appear to be no natural divisions. In the Pasteuri group the necessity for ammonium carbonate in the medium and resistance to high concentrations of this compound vary roughly with the urease activity of the organisms, but the latter property does not appear to be a consequence of a special tolerance of ammonia. In this work, a considerable number of bacteria, which do not produce a titratable alkalinity in urea bouillon, have been found to tolerate higher concentrations of ammonium carbonate than the weaker ureaclastic strains of the Pasteuri group.

THE GENERIC TERM UROBACILLUS

Urobacillus, introduced by Miquel (1888–89), has been used in a generic sense by Krogius (1890), Beijerinck (1901), Liebert (1909),

Orla-Jensen (1909), Söhngen (1909), Christensen (1910), Düggeli (1915), and Rubentschik (1926). In most cases the term was applied to any rod-shaped organism which decomposed urea actively, but Orla-Jensen proposed that the name be reserved for the spore-forming types. Rubentschik used the term *Urobacterium* for his non-sporing ureaclastic organisms.

If the prevailing tendency to multiply bacterial genera should persist, it is possible that the question of the recognition of a genus *Urobacillus* may be raised. It would be definitely unsatisfactory to include non-sporing organisms under this designation, for strong urease activity has been observed in various members of the Proteus, Coli-Aerogenes and Fluorescens groups, and in several chromogenic and other non-sporing bacteria. Among spore-forming bacilli the situation is almost as complicated, for the ability to decompose urea has been found in certain anaerobes (Tissier and Martelly, 1902), in occasional strains of *B. mycoides* and *B. megatherium* (Löhnis, 1910; Düggeli, 1915; Goeters, 1927), and in other organisms which do not require special media (Rochaix and Dufourt, 1910; Gibson, unpublished results). The genus *Urobacillus* might be restricted to include only the Pasteuri group and other ureaclastic aerobic spore formers which require or prefer alkaline, ammonia-containing media, but it is doubtful if these organisms constitute a series sufficiently well defined to merit generic status.

SUMMARY

Type cultures of organisms of the *B. Pasteuri* group were examined and compared with recently-isolated strains. The group may be divided into three moderately distinctive species: (1) *B. Pasteuri*, (2) *B. Loehnisii* n. sp. and (3) *B. Freudenreichii*. Spores are spherical or slightly ovoid in (1) and (2) and ovoid to elliptical in (3). Urea is decomposed rapidly by (1) and slowly by (2) and (3). The ability to decompose completely the urea in 2 per cent urea bouillon within forty-eight hours is suggested as the standard of urease activity distinguishing (1) from (2) and (3). Growth in ordinary neutral media is usually possible only for (2) and (3). Each species contains strains dissimilar in several

features and each is connected to the others by transitional forms. *Urobacillus Leubei* is regarded as a synonym of *B. Freudenreichii*. *Urobac. psychrocartericus* and *Urobac. hesmogenes* are probably synonyms of *B. Pasteuri*; and several other previously named bacteria appear to belong to this group. *B. sphaericus* and other organisms closely related to the Pasteuri group cannot be differentiated from the latter except by their inability to decompose urea or by their failure to show a preference for alkaline media containing ammonia. The suggestion is put forward that there is no group of urea-decomposing bacteria sufficiently well defined to deserve the generic term Urobacillus.

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THE MORPHOLOGY OF THE MYCOBACTERIA[1,2]

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The occurrence of branching forms of acid-fast bacteria was first emphasized by the classification of this group of microorganisms by Lehmann and Neumann in 1896 in their family "Hyphomycetes" and genus "Mycobacterium." Such a classification was based on the observation of supposedly filamentous and branched forms by Nocard and Roux (1887), Metschnikoff (1888), Maffucci (1892), Coppen Jones (1895), and Bruns (1895). These morphological studies were all made on organisms stained with carbol fuchsin. A minute accuracy is not possible under these conditions, since apparent branching may be due to a hazy outline of the cell boundaries; to capsular material staining continuously with the cell wall; and to the destruction of stereo-relationships so that it cannot be determined whether a cell lying at right angles to another cell is a branch on that cell or merely lying beside, underneath, or above it.

Miehe (1909) was the first to watch acid-fast bacteria developing in broth microcultures. He concluded that a saprophytic form, the "Harn" bacillus, did not branch, but that the human tubercle bacillus branched by budding, occasionally. In recent years controversy over the mode of reproduction of the human tubercle bacillus has stimulated several bacteriologists to watch acid-fast bacteria growing. Kahn (1929) believes that under given conditions—namely, single cells multiplying in microdroplets of a synthetic medium—the human tubercle bacillus reproduces by

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division into granules. These granules "sprout" into "delicate" rods, which in turn develop into "mature" rods. Gardner (1929) reports that human tubercle bacilli growing on serum agar blocks divide by binary fission only. Oerskov (1932), also using agar blocks and working with *M. phlei* and *M. tuberculosis*, says that the Mycobacteria are actinomycetes because they sometimes form a mycelium which later divides into segments or show an angular type of growth with frequent branching. Jensen (1934) divides the Mycobacteria into two subgenera on the basis of mode of cell division as observed by direct agar-microscopy, the one group, A, identified by the "snapping" and "slipping" growth, the other, B, exhibiting the "snapping" growth only. The "snapping" growth is the same as Oerskov's "angular" growth, and is defined by Jensen as follows: "a cell grows to a certain length, a line of division is formed at the middle, and the daughter cells bend suddenly into an angle, thereby producing V- or L-shaped figures. . . ." The following is his description of "slipping" growth: "after division, the ends of the daughter cells bend, slip past each other, and grow into parallel bundles." True branching occurs sometimes in the early stages in subgenus A; and there is a "transformation from branched rods to cocci" as growth progresses in subgenus B.

Micromotion pictures of the development of several members of the Mycobacteria, including *M. phlei*, tubercle bacilli from cold-blooded animals, and a non-acid-fast strain, have been made by Wyckoff and Smithburn (1933) and Wyckoff (1934a and b). Their findings are that these organisms multiply by binary fission in the active stages of cell division, that cocco-bacilli may be formed in aging microcultures, and that branching rods may be frequently found in cultures of the smooth types and occasionally in rough type growths.

Thus a survey of the literature leaves the reader with no clear-cut conception of a morphology characteristic of the members of the genus Mycobacterium. The factor preventing the organization of such a concept from the work of the various authors, is that the studies have been made using different methods of observation and under conditions for growth differing in respect to the

culture medium, moisture, and atmospheric oxygen. The personal factor of varying interpretations of similar observations, necessarily involved in microscopic work, also leads to confusion.

In isolating single cell strains of acid-fast bacteria by means of microcultures, we became interested in the growth phenomena; and in an attempt to clarify our own morphological concepts, made the following observations.

METHODS

Manner of mounting microcultures. The microcultures were made on thin number 1 coverslips mounted on hollow ground slides. The coverslips were cleaned with acid alcohol, and a thin coating of grease applied by dipping in a solution of vaseline in gasoline. They were then heated in a hot air oven at 160°C. for one hour, again cleaned in acid alcohol, and sterilized by heating in the oven at 160°C. for four hours. After this treatment there remains on the coverglass a very thin but adequate film of grease, the function of which is to prevent the droplets spreading. Droplets of glycerol broth were deposited around the edge of the depression of the slide in sufficient quantity to maintain saturated moisture conditions and in such a manner that there was no interference with the vision of the inoculated droplets. By proper regulation of the amount of grease on the coverslips and of the moisture conditions, the edge of the droplets will remain entire for several weeks. The coverslips were sealed on the slides with vaseline when incubation was carried out at room temperature, and with a half and half mixture of vaseline and paraffin when incubation was at 37°C.

Method of observation. Growth of individual organisms was followed by microscopic observations at such intervals of time as were necessary, depending upon the rate of division of the species under examination. A 1.8 mm. oil immersion objective and a 15× hyperplane ocular gave magnifications of 1425 times.

OBSERVATIONS

Culture. Mycobacterium 5-3A, smooth type. Mycobacterium 5-3A was isolated at the University of Wisconsin from the mesenteric lymph

node of a cow, but is non-pathogenic for rabbits, guinea pigs, and chickens.

Medium. Mannitol synthetic liquid medium (Dorset's formula with 1 per cent mannitol substituted for the glycerol[3]).

Method. By means of micropipettes controlled with a Chambers' micromanipulator, microdroplets of the medium containing one bacterial organism each were laid down in a circle around a loopful of the medium in the center of a coverglass. The bacterial suspension from which the micropipettes were filled was prepared from a forty-eight-hour culture of the bacteria in the mannitol synthetic broth. The slides were incubated at room temperature which varied from 22 to 25°C.

Microscopic examinations were made at intervals of twenty minutes. Since the division time of this organism is about five hours, the periodic examinations permitted following the fate of an organism and each of its daughter cells for approximately twenty-four hours, after which the number of organisms became so large as to cause confusion to the observer.

Data. Plate 1, figures 1 through 5, are representative of the results obtained. These particular drawings were selected from those depicting the development of about 50 single cells, as illustrating the points we wish to emphasize. Drawings did not happen to have been made in this case from the single cell stage up to the nineteenth hour, although observations were taken from the time of planting the single cell through the twenty-fifth hour at fifteen-minute intervals. We have deduced the following principles from these observations:

1. The organisms divide only by binary fission in young cultures.

2. The cells divide into two approximately equal parts. These two cells may, however, grow in length at different rates, but eventually attain about the same length. See cells 1*a* and 1*b* in figures 2, 3, and 4, and cells 4*a* and 4*b* in figures 2, 3, 4, and 5.

3. The rate of division depends upon some factor other than age and size of cell. See cell 7 in figure 2, and cells 7*aa*, 7*ab*, 7*ba*, and 7*bb* in figure 3. As corollaries to this principle, (a) rods of unequal size may divide. See cells 2 and 4 in figure 1, and 2*a*, 2*b*, 4*a*, and 4*b* in figure 2; (b) individual rods vary greatly in length (from 1.5 micra to 4 micra as shown by micrometer measurements).

[3] K_2HPO_4 ..... 1.0 gm. Ferric citrate..... 0.063 gm.
Tri-sodium citrate ..... 0.5 Asparagine..... 5.0
$MgSO_4 \cdot 7H_2O$ ..... 1.0 Glycerol..... 70.0 cc.
Dilute to 1,000 cc. with distilled water.

4. The cells grow in chains, the coherence apparently being due to the presence of capsular material. This capsular material can be demonstrated in macrocultures by Barlow's method of staining gum, as described by Fred and Waksman (1928).

5. The individual cells vary in shape. This seems to be due to two factors, (a) the cells are flexible (the idea of a rigid cell wall is apparently not true for young cells), and (b) as the cells grow in length, crowding occurs in the chain and due to the growth pressure the shape of the organism changes. These changes in shape are temporary, and vary with the passive growth movements in the chain. See cell 5, figures 2, 3, 4, and 5.

As the number of cells in the droplet increases, the chains break up into smaller and smaller chains. Finally when multiplication ceases, chain formation is entirely lost, and the surface of the droplet becomes filled with organisms, which, however, still lie in only one plane and show no tendency to pile up.

Culture. *Mycobacterium phlei*, smooth strain, Lister Institute Strain number 54.

Medium. Glycerol synthetic medium solidified with 1 per cent agar.

Method. Cells from a forty-eight-hour culture on the solid medium were separated by shaking with glass beads, suspended in liquid medium and a loopful of this material added to a tube of the melted agar medium. A loopful of this inoculated agar was placed on the coverglass. The slides were incubated at room temperature and examined at intervals of an hour.

Data. Essentially the same phenomena were noted as in the case of Mycobacterium 5-3A. There was more curvatuve of the rods, sometimes almost corkscrew-shaped rods being seen, since the agar probably somewhat prevented the freedom of the passive movements due to growth pressure. Again, growth proceeded until it had covered the surface of the drop. The organisms are of many bizarre shapes at this stage due to the fact that they are fitted together with little unoccupied space. Examples of the shapes seen are shown in figure 6 which represents a three months' old growth. These polymorphic shapes are much more numerous in an agar medium than in a liquid medium, and may be due to the agar obstructing the displacement of one cell by the growth movements of another cell. The organisms develop at the surface of the agar, but one does not know whether they are growing in the film

of moisture on the agar surface or just beneath the agar surface. Also, the surface moisture may contain some agar.

Coccoid forms as noted by Wyckoff and Smithburn (1933) were very numerous in certain areas along the edge of the drop, at the time when multiplication had ceased. These forms were oval, the majority measuring about 1.2 micra by 0.8 micron, while the majority of the rods measured about 3 micra by 0.6 micron. Such coccoid forms also result from transferring rods to flat microdroplets of fresh medium (the flatness of the droplet is regulated by the amount of grease on the coverslip). As Wyckoff and Smithburn point out, the length of the organism depends upon the relation between the rate of division and the rate of growth. Since the moisture and food in the flat droplets are more rapidly exhausted than in more convex droplets, it is probable that these are the determining factors of the rate of growth in length. One or two rod forms placed in such microdroplets divide, the second generation cells then divide after very little growth in length, and so on. The subsequent divisions thus result in the formation of smaller and smaller cells. The droplet becomes filled with cocci, which never grow any longer even after multiplication has ceased.

Culture. *Mycobacterium tuberculosis*, avian type, smooth strain.

Medium. Glycerol synthetic medium solidified with 1 per cent agar.

Method. The same procedure was used as for *M. phlei*, except that a ten-day old growth on Dorset's egg medium was used as the source culture. The incubation temperature of the slides was 37°C.

Data. It was found that the microcultures of this organism in the young active stages of reproduction could not be distinguished from those of M. 5-3A or *M. phlei*. A lag phase of three or four days occurred, but the generation time in the logarithmic growth phase of five or six hours was comparable to that of the other two cultures studied. However, after division ceased, the cells grew in length. Since the organisms lay too close together for accurate microscopic study, some of these long cells were removed with micropipettes to microdroplets of liquid medium. Figure 7 shows cells from a six weeks' growth. The curved rods did not straighten out when released from the pressure of

the other cells, so that apparently the cell wall becomes more rigid upon aging. Some of the rods were 8 to 10 micra long, but no branching forms were seen. These cells divided by binary fission and grew as usual when transferred to fresh loopfuls of the agar medium.

Microcultures of the rough strains of the above three organisms have also been observed. Their colonies are all of the wavy strand variety as illustrated by Wyckoff (1934a) in plate 28, figure 62, for the rough variety of *M. chelonei*. A "snapping and slipping" growth, as postulated by Jensen (1934) for his subgenus A, was observed in the initial stages of growth of single cells of all these rough strains. The fate of individual cells cannot therefore be followed, and since piling up begins very soon, the shapes of individual cells cannot be distinguished after the first few divisions. Also, the cell outlines are always more hazy than those of the organisms of the smooth types. However, from observations on the cells during the first multiplications and on individual cells picked up by pipettes from the main growth mass in the later stages of growth, their morphology and manner of division differ in no way from that of the smooth cells.

Culture. The human tubercle bacillus, Kahn's single cell strain of H37.

Medium. Glycerol synthetic medium solidified with 1 per cent agar.

Data. The microcolonies developing are of the rough type and are burr-like in form. Again, as for the rough strains of the other species, individual organisms cannot be discerned, but those separated out from the colonies by pipettes are not branched and do not differ in other ways in their morphology from the other species observed.

Single cells of the human tubercle bacillus placed in microdroplets of liquid synthetic medium, as in Kahn's method, have never grown.

DISCUSSION

Our observations on the dividing cells of three species of Mycobacteria may be summarized by saying that under the conditions of the experiments these organisms reproduced by binary fission only, and that there was no evidence of branching or any other life cycle form. Coccoid forms resulted from the occurrence of division at a time when there was very little growth in length;

and relatively long rod forms resulted when division had ceased but growth continued. The limiting factor in growth in length appears to be the conditions of nourishment, but a reasonable conjecture as to the limiting factor for division is not manifest. Forms deviating in shape from the straight rod with parallel sides are found in young cultures. These are temporary shapes and change as the pressure from neighboring cells varies, since the cell wall is flexible. More widely aberrant forms, probably so-called "involution" forms, are numerous in cultures when growth and multiplication have ceased and result from the cells being packed closely together. The cell walls become rigid upon aging, so that the shape of the cell is retained even when the pressure from other cells is released.

Since our conditions were comparable, why did we not see the branching forms reported by other workers? If preparations such as those illustrated in the first five figures are examined magnified 440 times instead of 1425 times, pictures such as that of figure 8 are seen. It is obvious by comparing the same preparation under the two magnifications, that the appearance of branching at the lower magnification is due to the flexibility of the cells and the slipping out of line of certain cells due to growth pressure in a chain—some of the spaces between the cells being too small to be evident unless magnified at least 1000 times.

We therefore deduce that the branching noted by Miehe (1909) and Jensen (1934), using magnifications of about 750 times, and Wyckoff (1934a) using magnifications of about 350 times, was only apparent. This deduction is further substantiated by the fact that the pictures given by these authors are similar to our picture in figure 8.

According to our data, the pseudo-branching was seen only in microcultures of the smooth types of the three species observed. Wyckoff (1934a) found the branching rods most frequently in smooth cultures, but occasionally in rough ones; and Jensen (1934) does not discriminate, reporting branched forms in both plane and perrugose variants. We believe that these "branched" forms in the rough cultures were really smooth types—either the

rough source culture contained some smooth forms, or dissociation of the rough to the smooth occurred in the microcultures. The possibility of these two explanations has been experimentally verified. Single cell cultures of Mycobacterium 5–3A, smooth type, when dissociating into the rough type, will show the presence of all proportions of smooth, mucoid, and rough types (as shown by plating out) depending upon the stage of dissociation. In microcultures in mannitol synthetic broth, single cells of M. 5–3A, rough type, will dissociate into the smooth type, unless the cell is deposited on the coverglass near but not in a droplet. Some small amount of liquid remains around the cell of course, and when multiplication has started, a growth of the rough type will spread over onto the droplet. Cells of the smooth type of the avian tubercle bacillus, on the other hand, will dissociate into the rough type in droplets of glycerol synthetic broth. It is therefore necessary to start the smooth growth in an agar medium. We do not wish to go into a discussion of dissociation here, however, but merely to indicate its relation to morphology.

CONCLUSIONS

1. No evidence was obtained, from our observations on microcultures of three species of Mycobacteria, namely a saprophytic species isolated at the University of Wisconsin, the phlei bacillus, and the avian tubercle bacillus, that division occurs by any method other than binary fission.

2. Branching forms were not seen when magnifications of 1425 times were used. Pseudo-branched forms were seen in cultures of the smooth type magnified 440 times.

3. The cells are not uniform in size or shape. Coccoid and relatively long rod forms were seen to be functions of the ratio of the rate of division to the rate of growth in length. Bent rods, club-shaped rods, and such types result from the flexibility of the walls of young cells and the growth pressures from other cells.

4. No difference was apparent in the morphology or mode of division of one species from another.

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PLATE 1

FIG. 1. The growth from a single cell of Mycobacterium 5-3A after nineteen hours from the time of "planting" in a microdroplet of mannitol synthetic liquid medium. The numbers have been arbitrarily applied to the cells as a means of identification.

Figs. 1 through 7 are drawings made at magnifications of 1425 times.

FIG. 2. Same as figure 1 after twenty hours. Cells 1*a* and 1*b* are daughter cells of cell 1 in figure 1, etc.

FIG. 3. Same as figure 1 after twenty-one hours. Cells 7*aa*, 7*ab*, 7*ba*, and 7*bb* are daughter cells of daughter cells of cell 7 in figure 2.

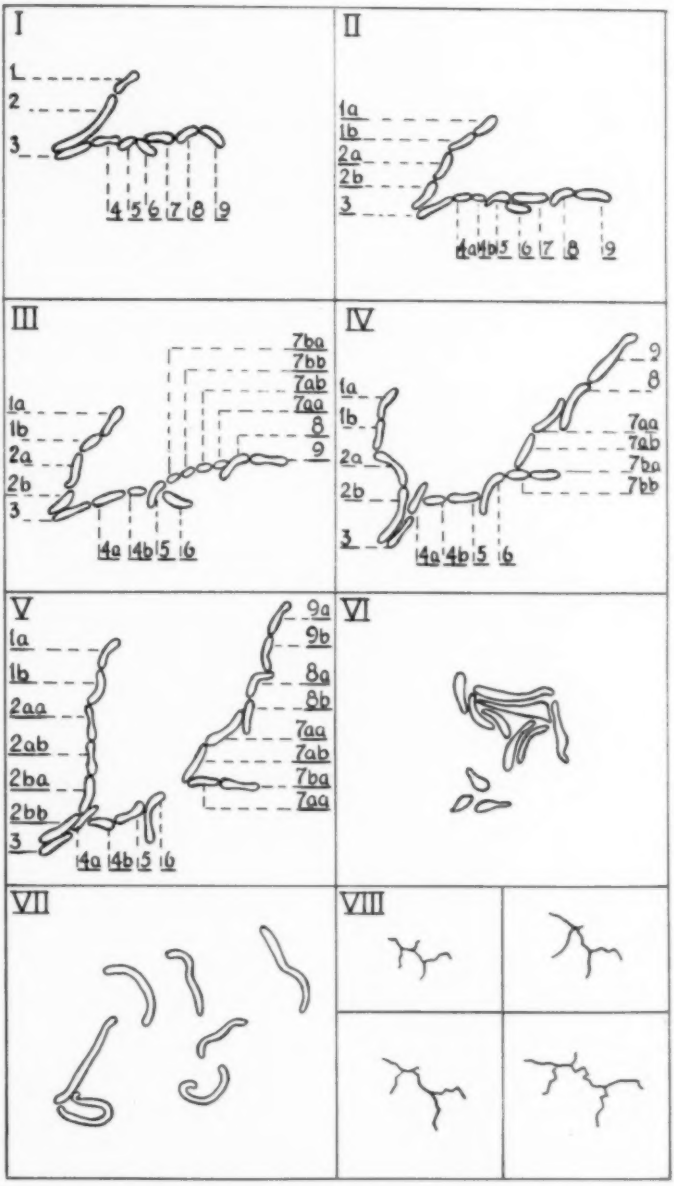
FIG. 4. Same as figure 1 after twenty-three hours.

FIG. 5. Same as figure 1 after twenty-five hours.

FIG. 6. Examples of cells of Mycobacterium phlei in a three months growth in a loopful of glycerol synthetic agar.

FIG. 7. Examples of cells of the avian tubercle bacilli in a six weeks growth in a loopful of glycerol synthetic agar.

FIG. 8. The growth from a single cell of Mycobacterium 5-3A after nineteen, twenty, twenty-one, and twenty-three hours respectively, in a microdroplet of mannitol synthetic liquid medium, as it appears when magnified 440 times.



(Janet McCarter and E. G. Hastings: Morphology of Mycobacteria)

STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

V. THE DIPHTHERIA BACILLUS

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In a preliminary report (Mueller *et al.*, 1933), evidence has been presented as to the nature of certain substances required for successful cultivation of a strain of diphtheria bacillus under investigation. It was there shown that a medium composed of meat extract, the amino acids formed on complete acid hydrolysis of casein, with tryptophane added, and a mixture of inorganic salts, permitted satisfactory growth. Omission of tryptophane resulted in failure to obtain growth; this amino acid was therefore essential for our strain or organism. Separation of the other amino acids by means of Dakin's (1920) butyl alcohol extraction method indicated that the unextracted residue as well as the "proline" fraction were essential. The only constituent of the former fraction shown at that time to be required, was cystine, which, when added alone to suitable control solutions, resulted in as good growth as when the whole crude fraction was used. Pure proline could not replace the crude proline fraction, so that another compound was inferred. Various attempts to isolate active, pure materials from meat extract failed, but it was shown that a non-basic substance, active in small amounts was involved.

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It is now possible to report more fully on the amino acid requirements, and it will be shown that excellent growth may be obtained by the use of a few pure amino acids together with meat extract and inorganic salts and some enriching substance. It is proposed immediately to undertake a re-examination of the meat extract component with the better methods now available and to be described here. A part of the findings to be detailed have been the subject of a brief preliminary note.[2]

GENERAL CONSIDERATIONS

Preparation of media

Media are prepared by dissolving the specified substances in water, (or in the case of cystine, in dilute HCl), and mixing in the proper proportions. Phenol red is added, in amounts of one drop of 0.2 per cent solution to each 10 cc. of completed medium, and the pH brought to 7.4 to 7.6 by addition of NaOH. Water is added until the desired quantities are contained in exactly 10 cc. of solution, and the media are tubed and sterilized at 10 pounds pressure for ten minutes.

Culture used

The same strain of diphtheria bacillus used in the earlier work has been maintained by daily transfer on meat infusion—"proteose" peptone broth, and kept at 36° to 37°. A small portion of the pellicle from such a culture after from eighteen to twenty-four hours is transferred to each tube of medium to be tested by means of a 3 mm. loop. It has not proved possible to inoculate more evenly by using a dilute emulsion of the organism, for growth frequently fails or is slow in becoming initiated. Moreover, growth on duplicate tubes is quite uniform with this method of inoculation. No attempt has been made to carry the organisms through repeated subculture on these simplified media, but naturally this will be done in due time. For the moment, the objective is strong growth in the first generation.

[2] Mueller, J. H.: Proc. Soc. Exp. Biol. and Med., 1934, **32**, 318.

Method of determining amount of growth

In our earlier work this was done by attempting to evaluate the thickness of the pellicle formed in twenty-four and forty-eight hours, and the results were recorded as − or ± to +++++. It became increasingly clear to us that this method was entirely unsuitable. With a series of media to be examined the difficulties of using such a rough, subjective method are obvious, and moreover, it is completely impossible to compare results over a period of time. Because of the nature of the mixtures used—commercial meat extract, and crude protein hydrolysates—cleanly negative controls could practically never be secured. It was felt that the amount of growth produced must be accurately determined, thus rendering possible the differentiations which it was necessary to make, in such a manner that results could be strictly comparable even over a period of months.

The method finally settled upon was a micro-kjeldahl determination of the bacterial nitrogen formed on a given amount of medium in a fairly definite time. The method has been already described elsewhere[3] and need not here be detailed. It is admittedly tedious in examining considerable numbers of cultures, but has so thoroughly proved its worth that we have no hesitation in urging that it be used by others working in this field, even if only on the basis that it permits of direct and easy duplication of results in various widely separated laboratories. Our tables and graphs are given entirely in terms of milligrams of total nitrogen present in the bacterial growth from exactly 10 cc. of culture medium, after an incubation period of fifty-five to sixty hours at 35°C. For the most part, each point plotted is the average of duplicate determinations. The tubes used are Pyrex test tubes with conical ends, described in the above citation, and they are incubated in a slanted position to allow adequate aeration and pellicle formation. Ordinary stock Pyrex test tubes of the same dimensions may be employed, but greater care is essential after centrifugation in pipetting off the supernatant, to avoid loss of bacterial substance.

[3] Mueller, J. H.: Jour. Bact. **29**, 383–388.

Planning of protocols

Our initial medium, which we wish to equal or improve upon, contains: Liebigs extract, 0.5 per cent; peptone 1.0 per cent; and NaCl 0.5 per cent.

This was simplified as to the peptone by substituting for that substance, casein acid hydrolysate, 1 per cent; and tryptophane, 0.01 per cent.

Tryptophane, normally present in casein and in enzymatic hydrolysates, is destroyed by strong acid hydrolysis and must be replaced. The amount used will later be shown to be adequate. There is no diminution in amount of growth obtained as a result of this substitution, certainly none for which adequate recompense cannot readily be made. The possibility of peptide linkages or other more complex effects than that of suitable amino acids is thereby practically removed.

Also from this point a salt mixture, with the following formula, is substituted for NaCl, which, in the presence of whole Liebigs extract may be unnecessary, but insures the presence of the commoner inorganic elements.

Salt mixture

NaCl	70.0 grams
K_2HPO_4	2.5 grams
$CaCl_2$	0.15 gram
$MgSO_4$	0.15 gram
$FeCl_3$	0.05 gram
HCl (concentrated)	0.5 cc.
H_2O to	350 cc.

0.25 cc. of this solution is added to each 10 cc. of medium

The twenty-odd amino acids comprising the casein hydrolysate were then divided roughly into three fractions by means of Dakin's (1920) butyl alcohol extraction method, viz: I. Monoamino fraction. II. Proline fraction. III. Unextracted residue.

These three fractions added in appropriate amounts in place of the casein hydrolysate practically reconstitute it, and likewise duplicate its effect. Of the three, fractions II and III were found to be essential in our earlier experiments while, fraction I was without marked effect. By omitting fraction I, using crude

fraction II and substituting the various amino acids present in fraction III, it has already been shown that cystine is the essential component, and in a concentration of 0.01 per cent which will later be shown quantitatively to be adequate, can replace fraction III.

This, then, was the situation at the outset of the work here to be described, and our attention was occupied in the attempt to replace fraction II in the following formula by a known amino acid or compound.

Liebigs extract	0.5 per cent
Tryptophane	0.01 per cent
Cystine	0.01 per cent
Salt mixture	
(Fraction II	0.1 per cent to 0.2 per cent)

This illustrates the method utilized throughout the work, namely to provide all the essentials for growth either pure or in crude form except the fraction under immediate examination. Cultures made on media lacking this fraction (controls), and the same controls plus various preparations to be examined constitute the experiment.

THE PROLINE FRACTION (FRACTION II, "P" FRACTION)

Using the above control solution and adding to it various "proline" fractions it soon became apparent that not all behaved alike. (It had already been shown that proline itself, alone, was without effect.) Some showed marked enhancement of growth over controls, others none. At this stage of the investigation quantitative methods of recording results had not yet been adopted.

Since the crude fraction contains traces of monoamino acids, separable only by repeated re-solution in absolute alcohol, additions of monoamino acid were again tried, although in earlier experiments they appeared evidently unnecessary. Now, however, almost invariable improvement in growth resulted. The concomitant presence of the proline fraction frequently but not invariably gave still better growth. In general, this fraction was prepared by distilling the butyl alcohol off in vacuum, dissolving the residue in hot absolute ethyl alcohol, cooling, and filtering

off the insoluble portion. A certain amount of water-insoluble sticky material was then removed by distilling off the ethyl alcohol, dissolving the residue as completely as possible in warm water filtering when cold, again distilling to a syrup and redissolving in absolute ethyl alcohol. The crude material thus obtained was ordinarily kept on hand dissolved in ethyl alcohol to prevent danger of bacterial contaminations and molds, and for use, the alcohol from a small portion was largely removed by warming on a watch glass over the water bath and redissolving in water. There seemed to be no reason to fear that the traces of alcohol remaining would influence growth, since they were too small to be inhibitory; however, the point was finally controlled, and it was quickly found that small quantities of alcohol considerably increased the amount of growth, quite evidently to an extent which appeared to explain the entire, (and irregular) effect of the "proline" fraction.

It was this finding which emphasized the necessity for a quantitative estimation of the amount of growth and the nitrogen method was worked out at this point. Table 1 and chart 1 show the effect of the addition of ethyl alcohol to the control medium. The rationale for the use of glutamic acid in the control solution will appear later.

Control solution (quantities given are for 10 cc. medium): 10 per cent Liebigs extract, 0.75 cc.; *l*-cystine, 1.0 mgm.; *l*-tryptophane, 1.0 mgm.; *d*-glutamic acid hydrochloride, 50.0 mgm.; "mono-amino" fraction, 50.0 mgm.; and salt mixture, 0.25 cc.

THE BUTYL ALCOHOL RESIDUE FRACTION (FRACTION III)

Glutamic acid

With a quantitative method available for estimating the amount of growth, it was learned that cystine did not account for the full effect of fraction III of the amino acids obtained by Dakin's method. Substitution of the pure amino acids other than cystine present in this fraction quickly demonstrated that the additional growth stimulus was supplied by glutamic acid. The other amino acids present were without effect in the controls as here constituted. It will be shown below, that one further com-

ponent of the fraction, namely histidine, must be supplied for maximum growth with further refinement of the medium.

The effect of the addition of glutamic acid in the form of the hydrochloride is shown in chart 2. In the interest of brevity the

TABLE 1

LOT NUMBER	COMPOSITION		NITROGEN
		cc.	mgm.
1	Control solution and ethyl alcohol	0.00	0.84
2	Control solution and ethyl alcohol	0.01	1.36
3	Control solution and ethyl alcohol	0.025	1.79*
4	Control solution and ethyl alcohol	0.05	1.87
5	Control solution and ethyl alcohol	0.06	1.92
6	Control solution and ethyl alcohol	0.07	1.84
7	Control solution and ethyl alcohol	0.08	1.91
8	Control solution and ethyl alcohol	0.09	1.78
9	Control solution and ethyl alcohol	0.1	1.55
10	Control solution and ethyl alcohol	0.125	0.95
11	Control solution and ethyl alcohol	0.250	0.58
12	Control solution and ethyl alcohol	0.50	0.35

* From this point the figures given are the averages of duplicate determinations.

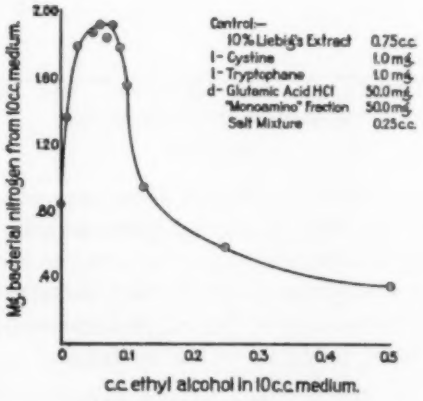

CHART I

actual figures need not be tabulated, but can be read with sufficient accuracy from the figure.

Alcohol in its optimal concentration is included in this and

subsequent media in order to push the maximum growth as high as possible so that the requirements for other factors may be more clearly demonstrated.

Tryptophane and cystine

Already recognized and reported as essential constituents of the medium, only qualitative information was available as to the amounts necessary for optimal growth. It was therefore deemed expedient to demonstrate quantitatively the effect of these two substances. The following curves (charts 3 and 4) are self explanatory, and indicate that the quantity of 1 mgm. per 10 cc.

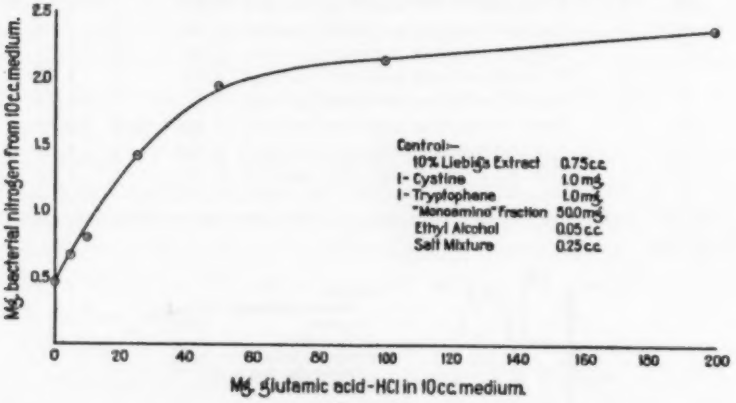

CHART II

used in earlier controls is probably about correct. The result is quite different from that shown by glutamic acid, and one may infer that the latter substance is used as a source of energy, whereas tryptophane and cystine probably each contribute an essential atom grouping, in the case of cystine, perhaps connected with the oxidation-reduction potential of the medium.

The "monoamino" fraction

Using, now, control media containing the above described factors, it may readily be shown that the presence of increasing quantities of the monoamino fraction causes a marked and progressive increase in growth. This is illustrated in chart 5.

In order to analyze this effect one must consider the amino acids known to be present in this fraction. These comprise the following: glycine, alanine, valine, leucine, serine, phenylalanine, methionine, tyrosine and oxyproline. Each of these amino acids was substituted for the monoamino fraction, but only a single

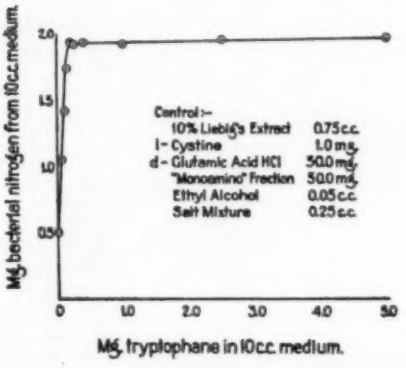

CHART III

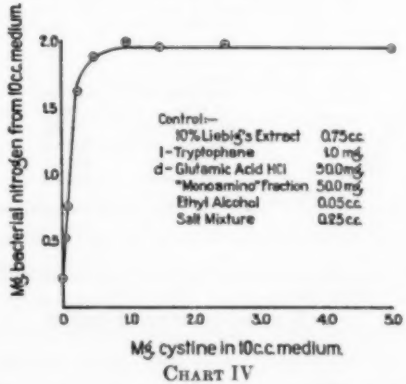

CHART IV

one, methionine, showed regularly any accelerating effect, and this a peculiar one, in that small amounts caused a marked increase in growth over the control, whereas, with even slightly greater quantities, the effect was lost. This fact is brought out in chart 6.

A considerable number of experiments such as the above have

been carried out, and the result is invariably the same. No material difference can be observed between the *l*- and the *dl*-forms. The latter is consequently used preferentially to rule out the possibility of the presence of unrecognized impurities. The curve starts up exactly as do those for tryptophane and cystine, but immediately flattens and descends with still a relatively low

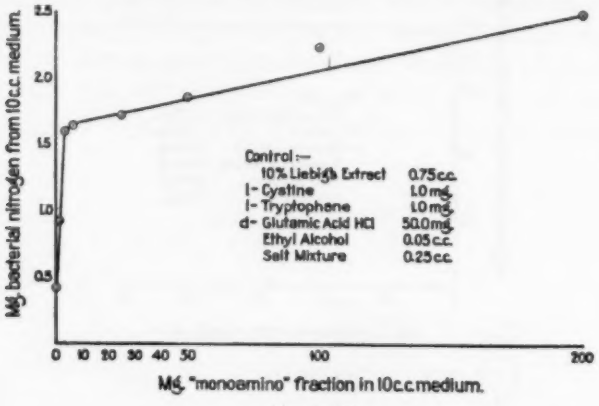

CHART V

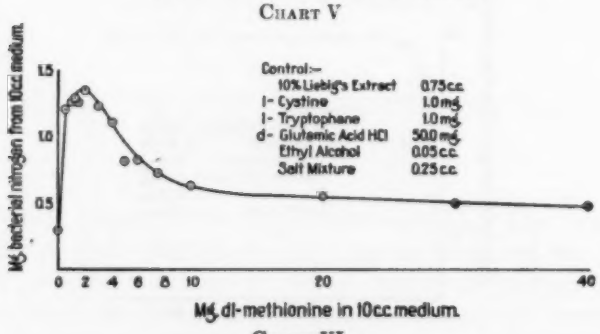
Mg. bacterial nitrogen from 10 cc. medium.
1.5
1.0
0.5
Control:—
10% Liebig's Extract 0.75 cc.
l- Cystine 1.0 mg.
l- Tryptophane 1.0 mg.
d- Glutamic Acid HCl 50.0 mg.
Ethyl Alcohol 0.05 c.c.
Salt Mixture 0.25 c.c.
0 2 4 6 8 10 20 40
Mg. dl-methionine in 10 cc. medium.

CHART VI

concentration of methionine, until it approaches the level of the control tubes. With even considerably larger amounts, however, it does not sink below this quantity, thus differing sharply from the effect shown by alcohol, which is of course inhibitory in moderate excess. Moreover, in the case of the addition of the whole monoamino fraction, which probably contains in the neigh-

borhood of 10 per cent methionine, it is clear that no such drop in the growth curve takes place, even in the presence of perhaps 15 to 20 mgm. of methionine (200 mgm. monoamino acid) in 10 cc. of medium. It would appear that methionine acts, in a manner quantitatively similar to tryptophane and cystine, by supplying a distinctive atom grouping, but only in the presence of some other compound, which occurs in traces in the control medium, probably in the Liebigs extract, and also in the monoamino fraction. The attempt to provide such a factor by the addition of any of the other known components of the monoamino fraction failed, either when these were used simply or in combinations.

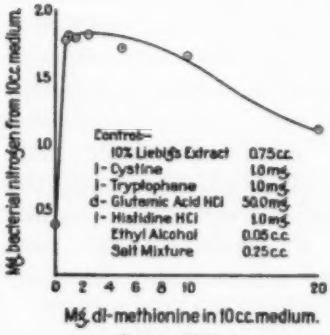

CHART VII

In various ways, the attempt was made to isolate from the monoamino fraction the substance responsible for this combined effect with methionine. It was finally learned that practically all of the desired material could be precipitated with silver oxide and baryta. This preparation, freed from silver and barium gave a marked Pauly reaction, and following this clue, histidine was tried, and proved at least partially effective as shown in chart 7.

Histidine has not been commonly recognized as being present in the monoamino fraction prepared by the butyl alcohol method, and has not actually been isolated from it in these experiments. Calvery (1931) has, however, demonstrated the presence of small amounts of histidine in the corresponding fraction prepared from

crystalline egg albumin, when extraction was continued as long as two hundred hours. It can only be stated that a substance in the "monoamino" fraction precipitable by regents ordinarily precipitating histidine, and giving the color reaction characteristic of imidazole compounds (among others) appears to exert a peculiar biological effect together with methionine, an effect which is approximated by histidine. The growth curve, however, even with considerable quantities of histidine, still shows a fall, after the maximum is reached. It may be stated parenthetically that histidine alone, without methionine, has absolutely no effect on the growth obtained from control media.

A mixture of the amino acids known to be present in the "monoamino" fraction (except methionine and histidine) was next prepared, each in approximately the proportion in which it occurs in casein. A total quantity of 50 mgm. of this mixture, added to 10 cc. of a control containing 2 mgm. methionine and 1 mgm. histidine hydrochloride gave a marked increase in the quantity of growth obtained. When used singly, and in corresponding amounts with the same control solution, the individual amino acids comprising the mixture gave no significant increases over the control alone. Evidently here again, more than a single substance was involved and it became necessary to eliminate one amino acid at a time from the total mixture. In this way serine and oxyproline proved to be without effect, alanine and particularly leucine appeared to be somewhat inhibitory, while glycine, valine and phenylalanine, when added together, considerably increased the quantity of growth, and tyrosine appeared either without effect or very slightly accelerating. The action of this rather troublesome mixture was next investigated quantitatively. The accelerating effect is most marked when all three are used simultaneously, and by varying the quantities it appears that 1 mgm. of *dl*-phenylalanine, 5 mgm. of glycine and 10 mgm. *dl*-valine will yield maximal growth. It has not seemed worth while to carry out sufficiently extensive experiments to construct curves demonstrating this fact, but chart 8 illustrates the effect of varying the quantity of methionine in the presence of these additional substances.

To bring together more clearly the results obtained with methionine, histidine, phenylalanine, glycine and valine, curves 6, 7 and 8 have been superimposed in chart 9.

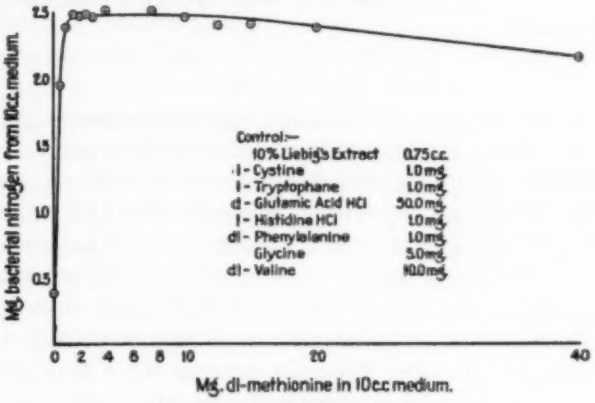

CHART VIII

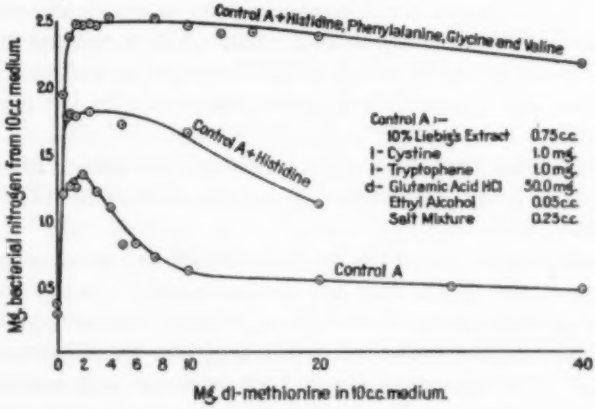

CHART IX

It is obvious that without methionine, the other four amino acids have little or no effect, since all the curves start at approximately the same level of nitrogen. It is evident, moreover, that this mixture of amino acids duplicates very closely the effect of

the crude "monoamino" fraction, in which a total of 2.5 mgm. nitrogen is reached with 200 mgm. of amino acids in 10 cc. (see chart 5), whereas the curve using pure amino acids rises sharply to this same value, flattens, and drops slowly as the quantity of methionine increases.

DISCUSSION

The nutritional requirements of various strains of diphtheria bacilli are known to differ widely. Braun (1930) speaks of "anspruchsvolle" and "anspruchslose" strains of this organism, and has succeeded in growing some of the latter on very simple media. Wheeler and Wadsworth, (1934) as well as others, have also cultivated certain strains and even produced toxins of some strength on synthetic media. It has happened that in our work we have evidently dealt with a strain having a fairly complicated list of requirements. Whether the daily passage for a long period of time on the usual peptone-infusion broth has gradually brought this about, or whether it was true from the first, we are unable to state. Experiments are now in progress in which the effect of each of the substances found of value with our strain is being tested on a number of freshly isolated strains, as well as on other old laboratory types. We hope to report shortly the results of this work.

From a somewhat different standpoint, it is also of interest to speculate as to the reasons for the various effects which have been brought out.

The ethyl alcohol is probably used directly as a source of energy. The two-carbon chain may be, for some reason, readily available to this particular organism. Thus, methyl and propyl alcohol are without effect, whereas acetic acid can be substituted for C_2H_5OH. On the other hand, both glycerol and maltose are effective but not enough work has been done to demonstrate how these substances compare quantitatively.

Glutamic acid, because of the considerable amount required, probably acts also as a source, either of energy or of nitrogen, or both. The presence of an associated impurity has not been excluded, but seems improbable because of the relative ease with

which this material is obtained in a high degree of analytical purity. It cannot be replaced by aspartic acid or asparagine, which indicates again a selective action on carbon chains of a definite length. We plan to investigate such similar compounds as glutamide, glutaric acid, etc.

Cystine, tryptophane, methionine, histidine and phenylalanine, producing their maximal effect at low concentrations, and each possessing a rather characteristic atom grouping, probably are required because of those groupings. The peculiar fall in the growth curve obtained with methionine, prevented to a considerable degree by histidine, particularly in the presence of, or when acting with, phenylalanine, glycine and valine, remains completely unexplained. The obvious suggestion is that, as a stage of its metabolism, some inhibitory substance is formed, but against this is the fact that growth never sinks below that of control tubes. It is quite probable that with the active investigation now being made in a number of laboratories of the part played by methionine and similar sulphur compounds in animal metabolism, an explanation for the phenomenon may be forthcoming. It is interesting that in animal metabolism methionine may take the place of cystine in artificial diets, whereas here both are required. It is perhaps also significant that methionine was first isolated during the study of a fraction of amino acids which made growth of a strain of hemolytic streptococcus possible, (Muller, 1922, 1923), although when purified, no effect could be demonstrated. The peculiar quantitative influence now recognized may account for that failure, or its presence may have been merely a coincidence.

That the medium as now constituted is at least the equivalent of a meat infusion-peptone broth is indicated by the fact that total bacterial nitrogen yielded by 10 cc. quantities of the latter is from 0.85 to 0.90 mgm., whereas our maximum is nearly three times as great, viz., 2.50 mgm. Naturally, an increased yield could be obtained from the bouillon if it were enriched by alcohol and glutamic acid. The writer feels, however, that by the use of the amino acids discussed in the present paper, the substitution for peptone in the bouillon has been adequately made. The

study is being continued in a re-investigation of the complex Liebig's Extract fraction, and it is hoped, by means of quantitative methods to arrive at a better understanding of the substances there involved than was possible in the preliminary investigations.

SUMMARY

It is shown that excellent growth of a rather fastidious strain of diphtheria bacillus can be obtained on a medium composed of Liebig's Extract, certain pure amino acids, ethyl alcohol and inorganic salts. The amino acids include *l*-cystine, *l*-tryptophane, *d*-glutamic acid, *dl*-methionine, *l*-histidine, *dl*-phenylalanine, glycine and *dl*-valine. The quantitative relations of these various compounds are presented, and their various actions discussed. The work is being extended to a further study of the meat-extract fraction and in other directions.

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DECOMPOSITION OF ORGANIC MATTER IN SEA WATER BY BACTERIA

I. BACTERIAL MULTIPLICATION IN STORED SEA WATER[1]

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The organic matter in the sea is unevenly distributed between the living plant and animal population, the suspended detritus, the organic matter in the sea bottom, and that present in the water in true and in colloidal solution. The living forms and the detritus make up only a small part of the total organic matter in the sea; the ratio between the organic matter in solution and that present in the total plankton was found to range from 7:1 (Gran and Ruud, 1926) to 300:1 (Krogh, 1934a).

The chemical nature of the organic matter in the marine bottom material was shown (Boysen-Jensen, 1914; Waksman, 1933) to be similar to that of humus in land soils, both in chemical composition and resistance to decomposition. However, the origin, chemical nature and transformation of the organic matter dissolved in the water are still open to question. According to Pütter (1907, 1924), algae give off into the water, in the process of their nutrition, large quantities of carbohydrates; it was suggested that this may possibly be a result of changes brought about by the bacteria adhering to the algae. Petersen (1911) was of the opinion that the dissolved organic matter does not come from plankton algae but from benthos formations. Pütter's ideas (1909) concerning the rôle of the dissolved organic matter in the nutrition of marine animals also met with considerable criticism.

[1] Contribution No. 57 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Krogh (1934a) found that the organic matter, as measured by the total carbon and nitrogen, is uniformly distributed in the sea, from the surface of the water downward; one cubic meter of water was shown to contain, on the average, 0.244 gram of nitrogen and 2.36 gram carbon. Although these results have not yet been confirmed and further extended, they point to certain important facts concerning the nature and distribution of the dissolved organic matter, namely: (1) the uniformity of its concentration in the water; (2) its specific chemical nature, as shown by a definite relation between the carbon and the nitrogen (about 10:1); (3) its non-availability as a nutrient for plant and animal life (it has even been considered as resistant to bacterial attack). Krogh (1930) emphasized that this organic matter must be looked upon as having gone out of organic circulation, thus representing slowly accumulating waste products; at least a fraction of it was believed to consist of "humus," highly resistant to further decomposition. The formation of stable organic compounds was also shown (Birge and Juday, 1934) to take place in the water of inland lakes, following the decomposition of plankton. The presence of true "humus acids" or their salts in solution in lake waters has been suggested by Ohle (1933).

A definite relation has been shown to exist between the transformation of the organic matter in the water and bacterial multiplication. When sea water is brought to the laboratory and allowed to remain undisturbed, rapid multiplication of the bacteria takes place (Gran and Ruud, 1926; Föyn and Gran, 1928); this results in the decomposition of a part of the organic matter in the water, accompanied by the absorption of oxygen (Knauthe, 1898; Fox, 1905; Winterstein, 1909; Harvey, 1925) and the evolution of carbon dioxide (Atkins, 1922) and ammonia. In addition to decomposition processes, bacteria also play an important function in synthesizing new complexes in the sea. Vernon (1899) found that in the purification of ocean waters by bacteria, a part of the ammonia is removed by these organisms and synthesized into cell substance. This has been confirmed recently by Butterfield and Purdy (1931), who look upon bacteria as important agents in absorbing dilute food from the water and concentrating it in their bodies.

The function of bacterial activities in the transformation of organic matter in sea water is thus shown to comprise two distinct processes: (1) the decomposition of the organic matter, resulting in the liberation of at least a part of the elements in forms (CO_2, NH_3, PO_4) available for diatom and algal nutrition; (2) the assimilation of some of the dissolved organic substances and their transformation into bacterial cells, which in their turn can be used as sources of food for marine animals.

EXPERIMENTAL

Course of bacterial development in stored sea water

The bacterial content of the water was determined by means of the plate method, which gives only the viable bacteria capable of developing on a specific medium. The results thus obtained represent only a fraction of the total number of bacteria, viable and dead, present in the water. A direct microscopic examination gave about 200 times as many organisms, the ratio becoming narrower upon addition of fresh organic matter. With due recognition of the limitations involved in the use of the plate method for measuring the abundance of bacteria in a natural substrate such as sea water, it was felt that this method is still most reliable for comparative purposes. An agar medium, previously found (Reuszer, 1933) to give good comparative results for determining numbers of bacteria in fresh sea water, was used. Several dilutions of the water were prepared and, whenever possible, only those plates which had 20 to 200 colonies were selected for counting. The plates were usually incubated for two to seven days, at room temperature (22° to 25°C.), unless otherwise stated. Oxygen was determined in the water by a modification of Winkler's method and ammonia by Krogh's method (1934b).

In a preliminary experiment on bacterial multiplication in stored sea water, samples of water were obtained near the dock at Woods Hole Harbor. The water was filtered through paper and kept in the laboratory exposed to light. Rapid multiplication of the bacteria took place, the numbers increasing from 85 cells in 1 cc. of fresh water to 1070 in twenty-four hours and to 157,000 cells within four days. It was soon found, however, that light

had an injurious effect upon bacterial development. In subsequent experiments, therefore, the water was kept in the dark, either in flasks plugged with cotton or in glass-stoppered bottles immersed under water.

The results of a typical experiment on bacterial multiplication in stored sea water, without and with the addition of different organic substances, are shown in figure 1. One portion of water was left untreated. One portion received a trace of an iron-ligno-

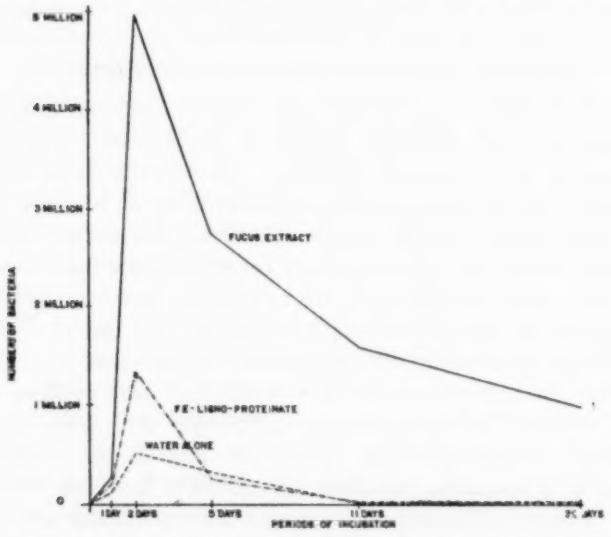

FIG. 1. INFLUENCE OF DIFFERENT FORMS OF ORGANIC MATTER UPON THE RATE OF BACTERIAL MULTIPLICATION IN SEA WATER

protein complex, introduced by adding 1 gram of this preparation to 500 cc. of fresh water, allowing to stand over night, and removing the undissolved organic matter by filtration through paper. A third portion received a small portion of a neutralized alkali extract of Fucus material. The rate of bacterial multiplication in stored water attained a maximum within very few days, usually 2 to 3; it then began to diminish rapidly, soon reaching a certain level or equilibrium which was considerably above that found in natural sea water. The amount and nature of the organic matter

in the water had a decided favorable effect upon the rapidity and extent of bacterial multiplication. Although the ligno-protein preparation was only sparingly soluble, it brought about an increase in bacterial numbers. The effect of the Fucus extract was still more marked, largely because of the greater availability of this form of organic matter, which offers an excellent source of energy for marine bacteria.

Among the different factors influencing bacterial activities and the rate of bacterial multiplication in sea water, temperature and oxygen concentration are most important. Two quantities of

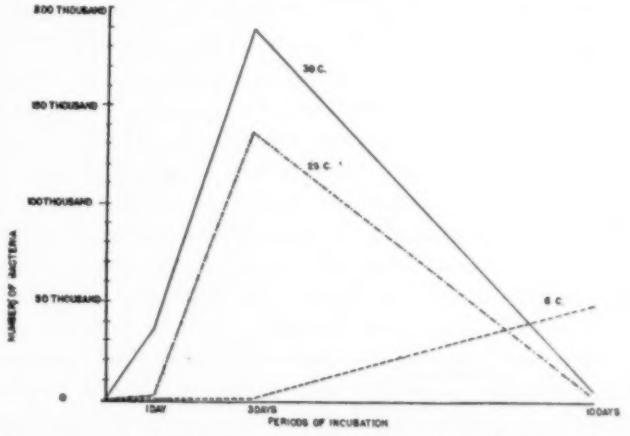

FIG. 2. INFLUENCE OF TEMPERATURE UPON THE RATE OF MULTIPLICATION OF BACTERIA IN STORED SEA WATER

water were collected from the middle of the Great Harbor at Woods Hole and about 1.5 miles off Quick's Hole, at a depth of 4 to 5 meters. The water was distributed into Erlenmeyer flasks, and these were incubated at three different temperatures, namely, 6°C., 25°C. and 30°C. At definite intervals, the numbers of bacteria were determined. The data reported in figure 2 represent averages of the results obtained for the two samples, since the numbers ran parallel in the water from both stations. The most rapid development of the bacteria took place at 30°C.; the maximum was attained within three days at this temperature, as well

as at 25°C. At 6°C., the rate of bacterial multiplication was very slow; however, the increase continued even after ten days incubation, so that on that day there were more bacteria in the water which had been kept at the lower than at the higher temperature, i.e., the maximum at the lower temperature was attained after a longer period of time. These results were confirmed by the use of water taken at a distance from shore.

The influence of depth of water upon bacterial multiplication, at 22° to 25°C., is shown in figure 3. There was an increase in the

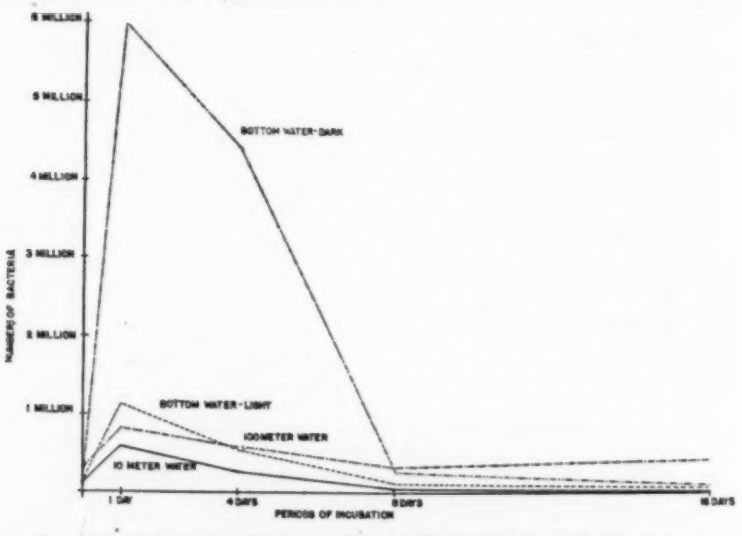

FIG. 3. INFLUENCE OF DEPTH OF WATER UPON THE RATE OF BACTERIAL MULTIPLICATION WHEN WATER IS KEPT AT 25°C.

rate of bacterial activities with an increase in depth of water. The actual numbers of bacteria found in the natural water were 2, 6 and 17 cells per 1 cc. of water, with increasing depths. The exceptionally high rate of bacterial multiplication in the water taken just above the bottom was no doubt due to contamination with some of the bottom material. The water exposed to light gave much lower numbers than the same water kept in the darkness.

Bacterial multiplication in filtered sea water

Bacterial development in stored sea water takes place at the expense of the organic matter suspended or dissolved in the water. The addition of a small amount of organic matter of a type comparable in chemical nature to that which is found in the sea has a decided stimulating effect upon bacterial multiplication. In order to throw light upon the specific nature of the organic matter in the water and its transformation as a result of bacterial action, three methods of attack suggest themselves, namely, (1) the separation of the organic matter by filtration of the water through different types of filters; (2) the study of the decomposition of the organic matter in water obtained from different depths, in relation to the photosynthetic zone; (3) the decomposition of different forms of organic matter added to the water.

Several methods of filtration were used in these experiments: (1) filtration through ordinary filter paper, hardened paper, or glass filters, thereby removing the coarser suspended particles and most of the plankton forms; (2) filtration through Seitz and membrane filters, which remove the bacteria as well; (3) colloidal filtration, thus leaving in the water only the substances present in true solution (Krogh, 1934b). The water filtered through Seitz, membrane and colloidal filters was always reinoculated with either fresh water or water kept for eighteen to twenty-four hours in the laboratory, whereby an enriched water culture was obtained. The vacuum produced in the filtration of the water through Seitz or membrane filters reduced the oxygen tension of the water, so as to make conditions unfavorable for the growth of the normal aerobic bacterial population commonly found in sea water; the water thus filtered was subsequently shaken for thirty to sixty minutes, to resaturate it with oxygen.

A quantity of water was obtained from the Great Harbor and separated into four portions: one was filtered through ordinary qualitative paper and is spoken of as fresh water; one was passed through a sinter filter; one was filtered through a Seitz filter; and one was ultra filtered. The water was inoculated and distributed in glass stoppered bottles; these were immersed in a water bath at 20° to 22°C. After definite intervals, the oxygen content was

determined in some of the bottles, while others were used for bacterial counts and ammonia determinations.

Only the results obtained for the fresh and sinter-filtered water are reported in figure 4. A definite parallelism was found to exist between bacterial multiplication, oxygen absorption and ammonia formation. In the case of the fresh water, 1.26 cc. of oxygen per liter was consumed in eleven days, while in the sinter-filtered water it was only 0.56 cc.; the corresponding amounts of ammonia produced in the two samples of water were 86 and 44

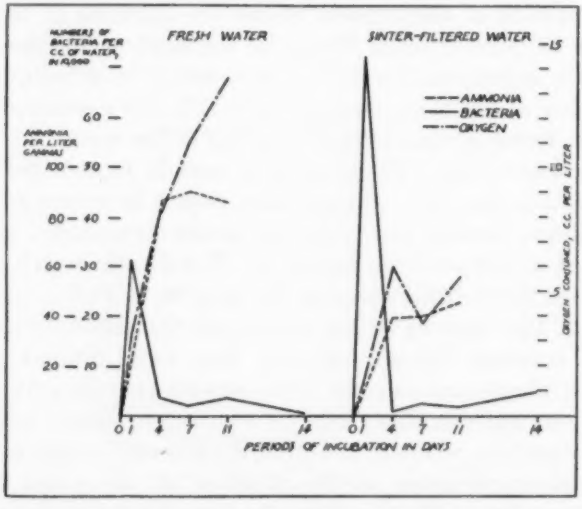

FIG. 4. INFLUENCE OF FILTRATION UPON BACTERIAL ACTIVITIES IN SEA WATER

gammas per liter.[2] This tends to show that the suspended material removed from the water in this experiment by sinter-filtration comprises a large part of the actively decomposing organic matter.

[2] The results of the ammonia determinations reported here should be considered as preliminary in nature. They were not checked sufficiently, hence no emphasis can as yet be laid upon them. In other experiments, either no increase in ammonia was obtained at all or only widely varying results were obtained. Although there is no doubt that in the process of decomposition of the organic matter in the water, nitrogen is liberated in an available form or in a form readily assimilable by bacteria as will be brought out later, the exact nature of this nitrogen is still a subject for further study.

Extensive bacterial multiplication took place in both the Seitz and ultra-filtered water; this was accompanied by oxygen absorption. In shaking the Seitz-filtered water, considerable ammonia was absorbed from the laboratory air, while only a small amount of colloid-filtered water was available; hence the results of bacterial activities in these portions of water are not reported; the fact was demonstrated, however, that the organic matter present in the water in true solution can be readily utilized by bacteria.

To determine the influence of the oxygen concentration of the water upon bacterial development, a quantity of fresh water was passed through a Seitz-filter. One-half of the filtered water was allowed to remain undisturbed, while the other half was thor-

TABLE 1

Influence of oxygen tension upon the activities of bacteria in sea water

TREATMENT OF SEITZ-FILTERED WATER	PERIOD OF INCUBATION							
	Start		2 days			5 days		
	Bacteria in 1 cc. of water	Oxygen	Bacteria in 1 cc. of water	Oxygen	Oxygen consumed	Bacteria in 1 cc. of water	Oxygen	Oxygen consumed
		cc. per liter		*cc. per liter*	*cc. per liter*		*cc. per liter*	*cc. per liter*
Aerated thoroughly.....	107	4.75	912,000	4.61	0.14	1,426,000	3.01	1.72
½ aerated + ½ unaerated...	88	4.42	9,350	4.37	0.05	1,098,000	3.54	0.86
¼ aerated + ¾ unaerated...	78	4.34	8,000	4.24	0.10	812,000	4.13	0.21
Unaerated.....	69	3.95	2,950	3.88	0.07	5,150	3.84	0.11

oughly shaken to saturate it with oxygen. Four quantities of water were now prepared: (1) filtered, unaerated; (2) filtered, oxygen saturated; (3) 50 per cent unaerated + 50 per cent aerated; (4) 75 per cent unaerated + 25 per cent aerated. These four lots of water were distributed into glass-stoppered bottles, which were then placed under water at room temperature. The oxygen content and bacterial numbers were determined immediately, and after two and five days incubation (table 1). Bacterial development in the unaerated water took place only very slowly; oxygen consumption was limited. In the fully aerated water, bacterial multiplication was normal, reaching over a million cells per 1 cc. within five days; this was accompanied by

rapid oxygen consumption. In the mixed water, the rate of bacterial multiplication was reduced by a decrease in oxygen content; however, a gradual adjustment of the bacteria to the diminished oxygen tension was observed. This modification of the bacterial activities obtained by reducing the oxygen tension of the water is of such distinct interest that, before the subject has been studied in further detail and before the modifications in the physical and chemical conditions of the water, such as change in hydrogen-ion concentration, are better understood, no broad generalizations can be made.

The results of another experiment, in which a greater variety of filters were used and in which an attempt was made to determine the effect of substances removed from the filter itself upon bacterial activities, are presented in table 2. The first few hundred cubic centimeters of the filtered water were discarded, and two subsequent portions used. The bacteria were again found capable of utilizing the organic matter present in the water in true solution. It is of interest to note that in the normal water and in the water modified by the addition of organic matter, as well as in the water incubated at different temperatures, there was a definite parallelism between bacterial multiplication and oxygen consumption, but not in the water filtered through fine filters. This may either be due to the development of specific bacteria which grow on the particular forms of organic matter left in the water, or to some specific effect of the filter upon the organic substances in the water. Attention is also called to the high numbers of bacteria in the water passed through the Seitz and colloidal filters.

The influence of depth of water upon bacterial multiplication and oxygen consumption is illustrated in table 3. In the case of the water obtained from the Gulf of Maine Station, the greatest activities took place in the water from 40 meters depth and then diminished above and below that zone. No such regularity was observed in the water taken from George's Bank, probably due to the constant mixing of the water at this station. Water was also obtained by means of Nansen bottles from station 2247 (latitude 39° 43′, longitude 69° 53′), to a depth of 1800 meters.

The greatest bacterial multiplication took place in the water taken at a depth of 50 meters, or within the photosynthetic zone;

TABLE 2

Bacterial activities in sea water passed through different types of filters

NATURE OF FILTER	BACTERIA IN 1 CC. OF INCUBATED WATER				OXYGEN CONSUMED ON INCUBATION		
	Start	2 days	5 days	7 days	2 days	5 days	7 days
					cc. per liter	*cc. per liter*	*cc. per liter*
Qualitative filter paper (No. 12)	810	1,145,000	73,000	15,400	0.84	1.37	1.46
Hardened filter paper	4,946	690,000	44,300	62,400	0.78	1.11	1.21
Seitz filter, first lot	650	210,000	942,600	432,000	0.82	1.34	1.43
Seitz filter, second lot	1,870	176,500	738,000	270,660	0.78	0.73	1.29
Membrane filter, first lot	846	705,000	23,000	42,700	1.17	1.02	1.05
Membrane filter, second lot	1,320	395,000	15,000	3,230	0.68	1.14	1.22
Colloid filter	1,800	940,000	712,000	621,000			
Seitz filtered sterile water (uninoculated)	0		0			0	

TABLE 3

Bacterial activities in sea water taken at different depths in the Gulf of Maine and George's Bank

POSITION OF STATION	DEPTH OF WATER	TEMPERATURE OF WATER	OXYGEN	BACTERIA IN 1 CC. OF INCUBATED WATER				OXYGEN CONSUMED ON INCUBATION		
				Start	2 days	5 days	10 days	2 days	5 days	10 days
	meters	*°C.*	*cc. per liter*					*cc. per liter*	*cc. per liter*	*cc. per liter*
Gulf of Maine 40° 34′ x 69° 19′	5	18.49	6.11	255	378,500	420,000	82,600	0.71	0.86	1.05
	40	5.72	6.23	343	647,500	205,000	185,000	0.55	1.26	1.33
	229	6.19	4.28	31	454,500	185,000	336,000	0.47	0.45	0.69
	5*	18.49	6.11	255	309,000	400,000	19,000	0.56	1.05	1.25
George's Bank 41° 10′ x 69° 30′	5	7.76	6.81	617	492,500	245,000	132,600	0.21	0.64	0.85
	20	7.64	6.62	525	388,000	70,000	143,000	0.37	0.46	0.76
	56	5.96	6.57	1,266	210,500	1,215,000	570,600	0.40	0.88	1.08
	5*	7.76	6.81	617	410,000	880,000	24,300	0.52		1.00

* Small amount of bottom material, mud or sand, added to each bottle.

water obtained at greater depths gave, on incubation, lower bacterial activities.

SUMMARY

A study has been made of bacterial multiplication in stored sea water, obtained from different depths and different regions, and modified by filtration through different filters. The results can be summarized as follows:

1. When sea water is placed in glass containers and stored in the laboratory or in the sea, rapid multiplication of the bacteria takes place.

2. The bacteria multiply at the expense of the organic matter present in the water in suspension and in solution, as measured by an increase in oxygen consumption.

3. The maximum development of bacteria takes place within two to three days, at 20° to 30°C. At lower temperatures, the rise in bacterial activities is much slower; however, in time they may attain as high if not a higher maximum than at the higher temperature.

4. The oxygen concentration of the water is highly important for bacterial activities; at a reduced oxygen tension, the organic matter of the water is attacked with considerable difficulty.

5. In the decomposition of the organic matter in the water by bacteria, a definite parallelism was obtained between bacterial multiplication, oxygen consumption and liberation of nitrogen in an available form.

6. These results lead to the conclusion that sea water contains sufficient organic matter in true solution, to support, under favorable conditions, an extensive bacterial population.

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DECOMPOSITION OF ORGANIC MATTER IN SEA WATER BY BACTERIA

II. INFLUENCE OF ADDITION OF ORGANIC SUBSTANCES UPON BACTERIAL ACTIVITIES[1]

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It has been shown previously (Waksman and Carey, 1935) that sea water contains sufficient organic matter in true solution to support an extensive bacterial population. It remained to be determined what forms of organic matter are more readily subject to bacterial attack and to establish the relation between bacterial multiplication and organic decomposition. Various organic substances, simple and complex in nature, were added to sea water and the response of the bacterial population determined.

In a preliminary experiment, some floating marine sediment collected by Dr. Stetson of this Institution was used. This sediment was obtained in Nantucket Sound (Hedge Fence), with a pan sitting 4 to 6 feet above the bottom; the total depth of the water was 12 feet. The dry material analyzed 18 per cent loss on ignition, 5.6 per cent organic carbon, and 0.68 per cent nitrogen, giving a carbon-nitrogen ratio of about 8:1. Two small quantities of this material, in a fresh state, equivalent to 72 and 87 mgm. of dry matter, were added to 200-cc. portions of water, previously kept for forty-eight hours in the laboratory in glass containers and the bacterial changes in which were known. The water was now again incubated at 20° to 22°C., for twelve days, and the bacterial changes determined (table 1). The results

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show that the sediment offers a favorable substrate for bacterial development.

UTILIZATION OF DIFFERENT ORGANIC SUBSTANCES BY BACTERIA IN SEA WATER

Freshly collected water was filtered through ordinary qualitative paper and divided into several lots. In order to eliminate the controlling effect of phosphate, all received, with one exception (control water), 10 mgm. K_2HPO_4 per 1000 cc. of water. Varying amounts of different organic substances were added, and the water distributed into a series of specially cleaned glass-stoppered bottles and flasks. The bottles, to be used for oxygen

TABLE 1

Influence of marine sediment upon bacterial multiplication in sea water

Bacteria in 1 cc. of water

PERIOD OF INCUBATION OF WATER	WATER ALONE	MARINE SEDIMENT, 72 MGM.	MARINE SEDIMENT, 87 MGM.
days			
Start	636		
1	144,000		
2	173,500	*	*
3	88,000	1,012,000	1,725,000
6	20,500	257,000	785,000
14	15,600	128,500	86,500

* Sediment added at this time.

determination, were incubated under water and the flasks, for the study of bacterial changes, in a dark closet (table 2). The fresh water contained about 600 bacteria in 1 cc. A normal course of bacterial development and oxygen consumption took place in the control water, the addition of K_2HPO_4 having comparatively little effect. The marine sediment was again found to favor bacterial development, as shown both by increased numbers and oxygen consumption. The glucose was attacked readily by the bacteria, but not as rapidly and completely as the alginic acid (as shown by the lower oxygen consumption). This is no doubt due to the fact that the latter contained a small amount of nitrogen as an impurity, while the glucose, being free

from nitrogen, brought about a nitrogen minimum as a result of the introduction into the water of a large amount of available energy. Fucus and its constituents, as well as the copepods and asparagine were utilized readily. The fresh diatom material was also decomposed rapidly; the lower numbers of bacteria and limited oxygen consumption were due to the fact that only an

TABLE 2

Influence of different organic complexes upon bacterial activities in sea water

NATURE OF COMPLEX ADDED PER LITER OF WATER	PERIOD OF INCUBATION					
	1 day	2 days		6 days		15 days
	Bacteria in 1 cc.	Bacteria in 1 cc.	Oxygen*	Bacteria in 1 cc.	Oxygen	Bacteria in 1 cc.
			cc. per liter		*cc. per liter*	
Control water	229,500	800,000	4.49	187,000	4.20	48,000
K_2HPO_4 alone	222,000	735,000	4.50	77,000	4.06	11,850
Marine sediment, 10 mgm.	970,000	975,000	4.37	695,000	3.55	
Glucose, 4 mgm.	1,135,000	1,070,000	3.98	430,000	2.41	470,000
Alginic acid, 4 mgm.	3,240,000	1,080,000	3.26	955,000	0.99	1,010,000
Cold water extract of Fucus, 10 mgm.†	4,520,000	3,815,000	0.84	1,135,000	0	
NH_4OH extract of Fucus, 10 mgm.‡	2,020,000	30,350,000	0	26,300,000	0	
Copepods, 50 Calanus	17,450,000	2,800,000	0.72	3,550,000	0	49,500
Asparagine, 4 mgm.	4,750,000	16,300,000	0	895,000	0	163,500
Fresh, dry Fucus, 10 mgm.	4,520,000	4,605,000	1.55	840,000	0	1,950,000
Pure culture of diatom suspension, equivalent to 3 mgm. dry matter	4,510,000	3,000,000	2.93	295,000	1.89	3,000

* The oxygen content of the freshly taken water was 5.10 cc. per liter of water.

† Dry matter basis.

‡ On basis of dry organic matter, most ammonia removed from extract by heating.

equivalent of 3 mgm. of dry diatom substance, representing 1.5 mgm. of organic matter, was added to each 200-cc. bottle of water.

Glucose was selected for a detailed study of the relation of bacterial multiplication to the decomposition of a known organic compound in sea water. When glucose alone is added to sea water, the available nitrogen soon becomes a limiting factor for bacterial multiplication; a small amount of ammonium sulfate

was, therefore, also added to some of the samples of water, at the rate of one part of nitrogen to 10 parts of glucose. Three different concentrations of glucose were added to portions of fresh, paper-filtered water, namely, 3.3, 10.0 and 26.7 mgm. per liter, distributed into glass-stoppered bottles and incubated under water at 20° to 22°C. At definite intervals of time, some of the bottles were removed and analyzed for bacterial numbers and for oxygen concentration. Because of the fact that these cultures were kept in the dark, algal growth was eliminated; further, because of a lack or only limited development of autotrophic bacteria, which could easily be demonstrated by the lack of formation of nitrite and nitrate, oxygen consumption in the water may be taken as an index of glucose decomposition.

The results presented in table 3 show that just about twice as much oxygen was consumed upon addition of glucose to the water, but without added nitrogen, as in the control water. The addition of larger amounts of glucose had no further effect upon oxygen consumption and very little upon bacterial multiplication. This is due to the fact that when glucose alone is added to the water, the bacteria depend for the nitrogen required for their metabolism only upon the organic matter normally present in the water. Hence, the decomposition of added glucose is controlled by the rate of the decomposition of the organic matter present in the natural water. However, when available nitrogen was also added, the bacteria decomposed much larger quantities of glucose, as determined both by the increase in oxygen consumption and by the rate of bacterial multiplication. A few determinations were also made of the ammonia content of the water. Although no quantitative data can be presented here, because the results were not checked sufficiently, they pointed definitely to the fact that while the bacteria in the unmodified water liberated appreciable amounts of nitrogen as ammonia, the glucose-containing water was free from ammonia; in the case of the water to which both glucose and a nitrogen source were added, rapid disappearance of the ammonia nitrogen took place.

Simultaneous with the decomposition of glucose in sea water, the decomposition of glutamic acid was also determined. In the

decomposition of 3.3 mgm. of this compound per liter of water, the bacteria consumed 1.92 cc. of oxygen in 3 days and 2.93 cc. in 7 days, i.e., exactly the same amounts as in the decomposition of a similar concentration of glucose plus available nitrogen. The rate of bacterial multiplication was also similar.

THE RELATION BETWEEN AVAILABLE NITROGEN AND GLUCOSE DECOMPOSITION IN SEA WATER

A further study of the decomposition of glucose in sea water brought out (table 4) the fact that sufficient nitrogen is liberated in one liter of water, as ammonia or as other available forms, to

TABLE 3

Bacterial activities in sea water receiving different amounts of glucose, with and without additional nitrogen

GLUCOSE ADDED	$(NH_4)_2SO_4$	BACTERIA IN 1 CC.				OXYGEN CONSUMED		
		Start	1 day	3 days	7 days	1 day	3 days	7 days
mgm. per liter	*mgm. N per liter*					*cc. per liter*	*cc. per liter*	*cc. per liter*
0	0	532	140,500	1,060,000	48,500	0.25	0.84	1.33
0	2.67	532			12,000			1.07
3.3	0	532	780,000	545,000	71,500	0.39	1.44	2.70
3.3	0.33	532		5,950,000			1.87	2.96
10.0	0	532	1,215,000	575,000	130,000	0.27	1.53	2.87
10.0	1.00	532		17,750,000	6,050,000		4.68	5.14*
26.7	0	532	1,370,000	480,000	96,500	0.39	1.35	2.82
26.7	2.67	532	14,600,000	7,100,000	515,000	3.62	5.14*	5.14*

* All oxygen in water consumed, anaerobic system.

enable the bacteria to decompose an additional 2.5 mgm. of glucose. The addition of nitrogen, in the form of ammonium sulfate, to the water had no further effect in bringing about any greater decomposition of the glucose, as long as the concentration of the latter did not exceed 2.5 mgm. per liter. When glucose was introduced in larger amounts, namely, 5 mgm. per liter, the addition of available nitrogen had a decided favorable effect upon its decomposition; however, in this case as well, only a certain concentration of nitrogen was required to favor the decomposition of the excess of glucose; any increase in the amount of ammonium sulfate added to the water had no further effect upon the process

of decomposition. When still larger quantities of glucose were added to the water, at the rate of more than 5 mgm. per liter, another limiting factor soon manifested itself, namely, the rapid consumption of the oxygen. Fresh sea water contains just about sufficient oxygen to bring about the complete utilization of 5 mgm. glucose per liter; however, only a part of the carbohydrate (about two-thirds) is completely oxidized to liberate energy, while another part is utilized by the bacteria for the synthesis of their cell substance. Larger amounts of glucose give rise to an anaerobic system which is no longer characteristic of normal sea water.

TABLE 4

Influence of nitrogen upon the decomposition of glucose in sea water

GLUCOSE ADDED	$(NH_4)_2SO_4$	BACTERIA IN 1 CC.					BACTERIA IN 1 CC., BY MICROSCOPIC METHOD	OXYGEN CONSUMED			
		Start	1 day	2 days	3 days	5 days	3 days	1 day	2 days	3 days	5 days
mgm. per liter	*mgm. N per liter*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*cc. per liter*	*cc. per liter*	*cc. per liter*	*cc. per liter*
0	0	0.883	625	775	295	121.6	65,000	0.69	0.80	0.96	1.28
2.5	0	0.883	2,005	2,450	675	44.3	80,000	1.31	1.76	2.13	2.49
2.5	0.25	0.883	3,850	3,700	735	9.6	120,000	1.21	1.62	1.82	2.41
5.0	0.25	0.883	8,850	8,150	1,125	84.0	107,500	1.98	2.36	2.93	3.58
5.0	0.50	0.883	6,900	7,200	880	57.6	82,500	1.81	2.16	2.47	3.41
7.5	0.75	0.883	12,550	11,700	1,740	13.6	162,500	2.63	3.09	4.10	4.76
15.0	1.5	0.883	24,050	29,000	4,200	349.6	220,000	4.22	4.86	5.28*	5.30*

* Oxygen practically all used up, anaerobic system produced.

In view of the extensive multiplication of bacteria in stored sea water, especially in the water enriched with glucose, the total number of bacterial cells can readily be determined by direct microscopic counts. The ratio between the numbers of bacteria obtained by the microscopic and plate methods was, in the case of the unmodified stored water, between 200 and 300, similar to the ratio previously found (Waksman, 1934) for bacterial numbers in sea water and in plankton tow. The water receiving glucose gave a lower ratio, which tended to approach 100:1 or even less. In the presence of an excess of readily available energy material and at an active stage of bacterial multiplication, there

was a greater proportion of viable bacterial cells, capable of developing into colonies on the plate.

The results presented in table 4 are sufficient to establish the relationship between the different bacterial processes, namely, decomposition of the organic matter in the sea water, bacterial multiplication, oxygen consumption and nitrogen liberation, the latter being determined by the extent of glucose decomposition (fig. 1). The consumption by bacteria of 1.28 cc. of oxygen per liter of water, in 5 days, is a measure of the decomposition of

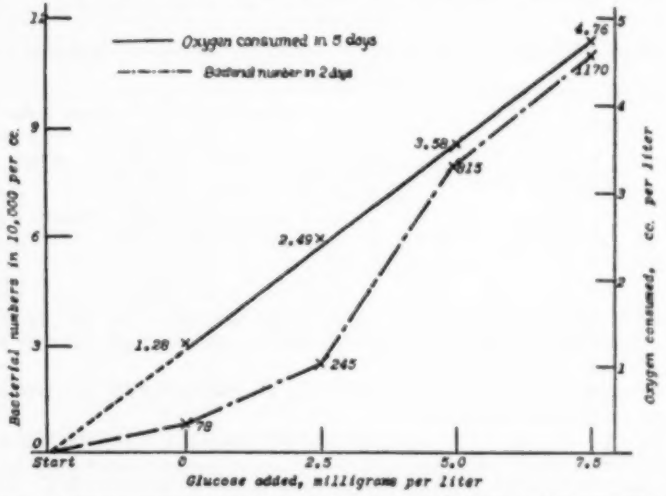

FIG. 1. DECOMPOSITION OF ORGANIC MATTER IN WATER, AS INFLUENCED BY ADDITION OF GLUCOSE

an equivalent amount of organic matter in the water, aside from that portion of the organic matter used by the bacteria for the synthesis of their cell substance; the latter may also be quite considerable, as shown by the microscopic counts. Using as a basis the results of Krogh (1934), namely that sea water contains about 5 mgm. organic matter per liter, it is a matter of simple calculation to determine that at least 25 per cent of the organic matter in the water is subject to rapid decomposition by bacteria; this is accompanied by the liberation of a corresponding amount of the nutrient elements (CO_2, NH_3, P_2O_5) stored in the organic

matter and essential for plant life. The bacterial cells synthesized in the process can obviously become a source of food for animal life.

The results of the following experiment serve to emphasize further the fact that the rate of glucose decomposition in sea water is a function of the liberation of nitrogen in an available form. A quantity of water was collected from the Harbor at Woods Hole, at a depth of 4 meters, filtered through paper and kept at room temperature, in the dark, for 4 days; another

TABLE 5

Decomposition of glucose in sea water as an index of liberation of nitrogen in an available form

NATURE OF WATER	BACTERIA IN 1 CC. AT START	NO GLUCOSE			GLUCOSE ADDED		
		42 hours	4 days		42 hours	4 days	
		Oxygen consumed	Bacteria in 1 cc.	Oxygen consumed	Oxygen consumed	Bacteria in 1 cc.	Oxygen consumed
		cc. per liter		*cc. per liter*	*cc. per liter*		*cc. per liter*
Water from harbor, fresh	925	0.79	129,500	1.21	1.56	660,000	3.45
Water from harbor, 4 days old	28,700	0.26	11,500	0.44	2.40	310,000	4.08
George's Bank water, 5 meters, 12 days old	560	0.25	16,000	0.44*	2.76	100,000	4.70
George's Bank water, 56 meters, 12 days old	14,200	0.34	551,000	0.37†	3.41	2,026,000	4.27

* Same water freshly taken consumed, when incubated at 20° to 22°C. in the darkness, 0.64 cc. of oxygen per liter in 5 days.

† Oxygen consumption of fresh water 0.88 cc. per liter in 5 days.

quantity of water was taken fresh from the same place in the Harbor and also filtered through paper. Two other lots of water were obtained from George's Bank, at a depth of 5 and 56 meters, and kept in glass-stoppered bottles for 12 days at 6°C. These four lots of water received 6.7 mgm. K_2HPO_4 per liter and each lot was divided into 2 parts, one not receiving any glucose and the other receiving 10 mgm. glucose per liter. These lots were distributed in glass-stoppered bottles and incubated, under water, at room temperature. Bacterial numbers and oxygen consumption were determined after 42 hours and 4 days. The results

(table 5) show that the water taken from the Harbor and stored for 4 days, at room temperature, liberated sufficient nitrogen to enable the bacteria to increase considerably the rate of glucose decomposition. The same is true of the water from George's Bank. Since this water had been incubated for 12 days before glucose was added, a much greater amount of the carbohydrate was decomposed without the addition of nitrogen.

TABLE 6

Influence of temperature upon bacterial activities in sea water

TEMPERATURE	GLUCOSE ADDED*	NUMBERS OF BACTERIA						OXYGEN CONSUMED			
		Start	1 day	3 days†	6 days	9 days	12 days	1 day	3 days	6 days	9 days
°C.		*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*cc. per liter*	*cc. per liter*	*cc. per liter*	*cc. per liter*
2	−	0.85		5.2	172.5	1,215.0	529		0.04	0.26	0.45
12	−	0.85	3.3	492.0	677.0	87.5		0	0.52	0.83	0.94
22	−	0.85	438.0	492.0	94.0	6.2		0.49	0.85	1.39	1.54
34	−	0.85	539.0	581.0	55.0	37.0		0.43	0.67	1.14	1.18
2	+	0.85		5.5	175.5	1,135.0	762		0.04	0.21	0.56
12	+	0.85	5.2	775.0	550.6	140.0		0	0.80	1.55	2.29
22	+	0.85	1,355.0	1,130.0	265.0	122.5		0.72	1.82	3.87	4.77
34	+	0.85	1,110.0	600.0	356.0	504.0		1.64	3.65	4.25	4.80

* Ten milligrams per liter of water.

† For the 2°C., read 2, 5, 8, 12 days.

INFLUENCE OF TEMPERATURE OF INCUBATION UPON BACTERIAL MULTIPLICATION AND OXYGEN CONSUMPTION IN WATER

Water obtained from Woods Hole Harbor was filtered and divided into two lots, one of which received 10 mgm. glucose per liter. Incubation took place in glass-stoppered bottles, in special water baths, kept at different temperatures. The results obtained in this experiment (table 6) tend to confirm the observations previously made, namely, that at higher temperatures, bacterial multiplication in stored sea water proceeds most rapidly and soon attains a maximum; this is followed by a rapid drop in numbers. At lower temperatures, however, the bacteria begin to develop more slowly but in time they attain as high a rate of activities as that observed at the higher temperatures (fig. 2). Similar

results were obtained for bacterial multiplication in river water (Butterfield, 1933).

DISCUSSION

Bacteria are largely responsible for the mineralization of the organic residues of plant and animal origin in the sea; the energy thus liberated enables them to synthesize new complexes, largely in the form of bacterial cells or their decomposition products, which are found in the water in the form of suspended matter as well as in true solution. The organic matter in sea water is in a

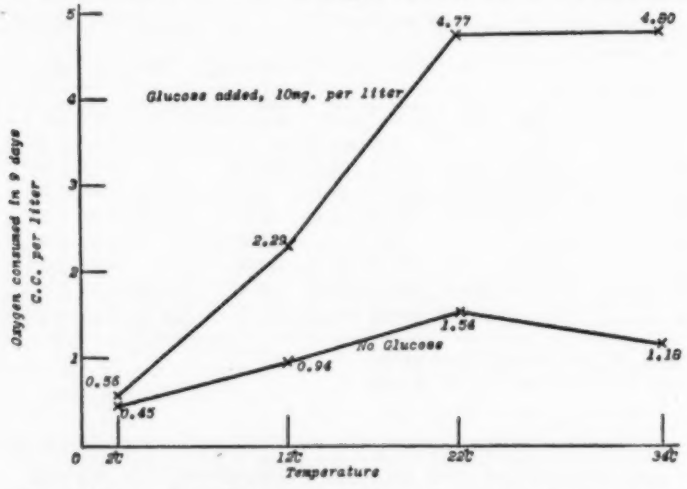

FIG. 2. INFLUENCE OF TEMPERATURE UPON BACTERIAL ACTIVITIES IN SEA WATER, AS MEASURED BY OXYGEN CONSUMPTION

state of dynamic equilibrium in which the residues and waste products of higher forms of life, bacterial activities and the dissolved organic complexes, are all involved.

Although the concentration of organic matter in sea water is small, namely about 5 mgm. per liter, as determined from the results of Krogh, it is not absolutely resistant to bacterial decomposition. When water is enclosed in a vessel, active bacterial multiplication sets in; this is accompanied by rapid decomposition of the organic matter. The rapidity of this process depends upon

a number of factors, especially temperature, oxygen tension, abundance of organic matter and chemical nature of the organic matter itself. The determination of the rate of bacterial multiplication in sea water is the most sensitive index for the study of the changes produced in the organic matter content of the water as a result of bacterial activities; a slight change in organic matter concentration brings about an extensive change in the numbers of bacteria. This method can be supplemented by the determination of the biochemical changes in the water, especially oxygen absorption and liberation of nitrogen as ammonia.

Most bacteria present in sea water are aerobic in nature; sea water contains sufficient oxygen to permit oxidation processes to proceed uninterruptedly in the presence of concentrations of organic matter comparable to those ordinarily present in the water; the amount of oxygen consumed by the bacteria in the process of oxidation must, therefore, be parallel to the amount of CO_2 liberated. Since the organic matter of the water is of a more or less constant carbon-nitrogen ratio, as indicated by the results of Krogh, the decomposition of a certain amount of this organic matter should be accompanied by a parallel liberation of nitrogen, as ammonia or in some other available form. Since the decomposition of glucose added to sea water was found to be controlled by the amount of available nitrogen in the water, the liberation of nitrogen in the decomposition of organic matter in the water can be determined not only directly but also indirectly, namely by adding glucose to the water and measuring the rate of its decomposition.

A definite parallelism was obtained between the increase in bacterial numbers, oxygen absorption and liberation of nitrogen in an available form in stored sea water. This parallelism was modified considerably by the separation of the organic matter in the water by means of different filtration procedures. Water passed through a filter fine enough to remove the bacteria and reinoculated with cultured fresh water gave a higher rate of bacterial multiplication; the latter was not always accompanied by a parallel increase in oxygen consumption. Whether this was due to the modification of the organic matter by the filtration

process, whereby the development of special groups of bacteria was favored, or to some other factor still remains to be determined.

The results presented here are sufficient to permit calculation of the relationship between bacterial development and organic matter transformation in sea water. Although bacterial multiplication and oxygen consumption run parallel in stored sea water, they are not directly proportional, bacterial multiplication preceding somewhat the consumption of the oxygen. The number of active bacteria varies considerably during the period of active multiplication, followed by their gradual destruction; the amount of oxygen used by a certain number of bacteria is not the same throughout their life cycle. The results presented in table 4 can be taken as a basis for computation. Without the addition of glucose, the bacteria increased from 883 to 625,000 in 1 cc. of water, in 24 hours; this was accompanied by the consumption of 0.69 cc. oxygen per liter of water, or 1.1 cc. of oxygen consumed per liter for an increase of 1 million bacteria per 1 cc. During the second 24 hours, the bacterial numbers increased by 150,000 and the oxygen consumption by 0.11 cc., or 0.73 cc. oxygen was consumed per liter for an increase of 1 million bacteria in 1 cc. The maximum rate of bacterial multiplication seems to have been reached about that time; the numbers later began to diminish rapidly, since, within the third day, there was a drop in numbers of 480,000; this drop was accompanied by an increase of 0.16 cc. oxygen consumption per liter. During the following two days, the bacterial numbers dropped by 173,000, while the oxygen consumption continued at the rate of 0.16 cc. per liter per day.

When glucose was added to the water, at the rate of 2.5 mgm. per liter, there was just about twice as much oxygen consumed as in control water; the rate of bacterial development was also higher, especially during the first three days of active multiplication. The consumption of 1.28 cc. of oxygen per liter of water indicates the complete decomposition of about 30 per cent of the organic matter naturally present in the water. Assuming a stable relation of the carbon and nitrogen in the water, one would expect that about 30 per cent of the nitrogen in the water, or about 75 gammas per liter, should also be made available. This amount

of nitrogen is just sufficient for the complete decomposition of 2.5 mgm. of glucose, since the addition of available nitrogen had no effect in bringing about any greater decomposition of this amount of glucose; the assumption is therefor made that all the glucose is decomposed in 5 days, an assumption fully justified because of the aerobic system of the sea water and because bacterial numbers have already passed their maximum. In the presence of 2.5 mgm. of glucose, there was an increase in oxygen consumption of 1.21 cc. per liter, which is equivalent to about 1.5 mgm. of glucose; one may, therefore, conclude that only about 60 per cent of the glucose has been completely oxidized and the energy utilized for bacterial activities. The other 40 per cent of the carbohydrate had no doubt been changed largely into bacterial cell substance, as can be demonstrated by theoretical considerations: 1 mgm. of glucose contains 0.4 mgm. carbon; the carbon-nitrogen ratio of the bacterial cell is about 5:1; this would mean that the synthesized bacterial cells should require about 80 gammas of available nitrogen per liter, a figure close, if not identical, to that calculated from the decomposition of the organic matter in the water.

The above calculations are fully confirmed by the results obtained with increasing amounts of glucose. The addition of 5 mgm. of glucose per liter of water brought about the consumption of 3.58 cc. of oxygen in 5 days; by subtracting the amount of oxygen consumed in the control water, a difference of 2.3 cc. is obtained; this is just twice as much as the oxygen consumption in the decomposition of 2.5 mgm. glucose. The corresponding amount of oxygen used by the bacteria in the decomposition of 7.5 mgm. glucose was 4.76; allowing for the control water, it gives 3.48 cc., or just about three times as much oxygen consumed as for 2.5 mgm. of glucose.

These data (fig. 1) are sufficient to emphasize not only the parallelism in oxygen consumption and glucose decomposition, but also that between oxygen consumption and decomposition of organic matter present in natural sea water. When only 2.5 to 7.5 mgm. glucose were added per liter of water, the oxygen did not become a limiting factor. Further addition of nitrogen had no

effect in bringing about any greater oxygen consumption, which leads one to conclude, especially since bacterial numbers were already diminishing rapidly, that all the glucose in the water had been used up. If the straight line relationship is true for the decomposition of the glucose added to the water and the oxygen consumption, there is no reason why it should not hold true for the decomposition of the organic matter of the water itself and oxygen consumption. An amount of organic matter, comparable to about 2.5 mgm. of glucose, or about 50 per cent of the total organic matter in sea water, is thus found to be readily subject to decomposition by bacteria. About 60 per cent of the organic matter decomposed was completely oxidized, as shown by the amount of oxygen consumed, and about 40 per cent of it was converted into bacterial cell substance and other products of bacterial metabolism.

Similar results were obtained in other experiments, as reported previously (Waksman and Carey, 1935, fig. 4). The oxygen consumed by the bacteria in the fresh water, in 11 days, was 1.26 cc. per liter; in the case of the sinter-filtered water, only about a half as much oxygen, or 0.56 cc., was consumed. The liberation of ammonia was parallel to the consumption of oxygen and confirms the above theoretical calculations. The liberation of about 80 gammas of ammonia per liter also points to the fact that about a half of the organic matter in the fresh water is subject to decomposition, since some of the nitrogen thus made available will be reconsumed by the bacteria.

The most important and crucial question may now be raised. Since extensive bacterial multiplication can take place in sea water, when some of it is enclosed in a glass vessel and allowed to remain undisturbed, why does the same process not occur in the sea itself, under natural conditions? No definite answer can as yet be given to this question until more is known of the factors controlling and favoring bacterial development in the sea. One may suggest, however, the following hypothesis: Under natural conditions, the organic matter in the water is in a state of equilibrium between formation and decomposition; this equilibrium may be considered as dynamic rather than static. New organic

complexes are continuously formed in the water as a result of the decomposition of plant and animal residues; this tends to compensate for the gradual destruction of the organic matter due to bacterial activities. The bacterial population in the sea is kept down, under natural conditions, to a certain minimum, due, on the one hand, to the consumption of the bacteria by protozoa, copepods and other marine animals and, on the other hand, to the presence in the water of "certain controlling factors" injurious to free bacterial development. When sea water is placed in a glass container, the animals rapidly die out; any modification of the other controlling factors, whether organic or inorganic in nature, is still a subject for further study. These modifications, however, are sufficient to enable the bacteria to begin to multiply rapidly and utilize the organic substances present in the water both in suspension and in solution. The relatively large amount of organic matter found in solution in sea water may, therefore, not be due merely to its absolute stability, but to the fact that the specific conditions are not favorable for rapid bacterial multiplication. When more favorable conditions are created, as in the process of constant mixing of the water, greater bacterial multiplication and greater oxygen consumption takes place; one has to compare only the activities of the bacteria in the water on George's Bank with those of the Gulf of Maine, to find a certain justification for this assumption.

The specific nature of the controlling factors which prevent more abundant bacterial multiplication in the sea itself may vary. It is known that as a result of continuous growth of bacteria in the same liquid medium, products are formed which are unfavorable to their further development. Frequently the mere aeration of the culture is sufficient to favor bacterial multiplication. A further illustration is found in the case of land soils; when a soil is brought to the laboratory and mixed, much more abundant bacterial multiplication takes place than in the undisturbed soil; after a certain period of time, a new equilibrium is established.

The bacterial activities in sea water may thus be considered to be in a state of dynamic equilibrium. Any modification of this equilibrium, the exact conditions of which are not yet understood,

brings about a rise in bacterial activities; this is accompanied by a rise in the rate of decomposition of organic matter in the water. This organic matter can, therefore, not be considered as absolutely resistant to bacterial action, but its presence in the sea is merely a result of specific conditions which control the bacterial equilibrium. When these conditions are modified and bacterial development favored, the organic matter can undergo rapid decomposition.

SUMMARY

1. The amount and specific nature of the organic substances added to sea water control the abundance of the bacterial population developing in the water.

2. The decomposition of a nitrogen-free organic material, such as glucose, added to the water is controlled by the amount of available nitrogen present in the water.

3. As a result of the decomposition of the organic matter present in sea water, some of the nitrogen is liberated in an available form; this can be measured not only directly as ammonia, but also indirectly, by the extent of decomposition of glucose added to the water. Any excess of glucose remains unattacked, unless some available nitrogen is also introduced.

4. When sea water is placed in glass containers and kept under favorable conditions, a large part (25 to 50 per cent) of the organic matter in the water is decomposed within 10 to 12 days, as measured by the amount of oxygen consumed and nitrogen liberated in an available form.

5. A tentative explanation is suggested for the stability and uniformity of the organic matter in sea water, based on the specific relationship of bacteria to the animal population of the sea and to the presence, under natural conditions, of factors unfavorable to rapid bacterial development.

In carrying out these investigations, the authors were assisted by several members of the Oceanographic Institution to whom grateful acknowledgment is made here. Dr. Ancel Keys obtained the samples of water from the deep sea station and prepared the

ultra filtered water; he and Mr. Davis have also made the ammonia determinations. Dr. David Kirsh and Mr. C. E. Renn assisted in obtaining the samples of water and in making the oxygen determinations.

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IDENTIFICATION OF ASPARAGINE AS THE SUBSTANCE STIMULATING THE PRODUCTION OF BUTYL ALCOHOL BY CERTAIN BACTERIA[1]

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In a previous paper, Tatum, Peterson and Fred (1934) described a factor found in potato and potato extract which markedly stimulated the fermentation of starch and the production of butyl alcohol by certain butyric acid bacteria. The stimulant was found in many other plant materials and varied in its effect on different strains of the bacteria.

Since this publication the active principle has been isolated from potato and identified as *l*-asparagine. Both *l*-aspartic and *d*-glutamic acids have an equivalent stimulatory action, while other related compounds, ammonium malate and ammonium succinate, cause a definite but less effective stimulation.

EXPERIMENTAL

Isolation and identification of the active principle

As an index to the presence of the active principle during the isolation, culture 21 (McCoy, Fred, Peterson and Hastings (1930)), was used, since this culture gives a maximum and consistent response.

The fermentations were carried out in test-tubes containing 35 cc. of 4 per cent corn mash. The cultures were analyzed for butyl alcohol (Johnson (1932)), the amount of which is an accurate index of the degree of stimulation.

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

Method of isolation. The method of isolation (fig. 1) involved treatment of the potato juice with lead acetate and ammonium hydroxide to remove carbohydrates, proteins, etc. The ammonia was removed by aeration from alkaline solution, and the active principle precipitated by mercuric acetate and sodium carbonate (Neuberg and Kerb (1912)). The mercury was removed from the precipitate with H_2S and the excess H_2S by aeration. Evaporation of the solution led to the crystallization of an active

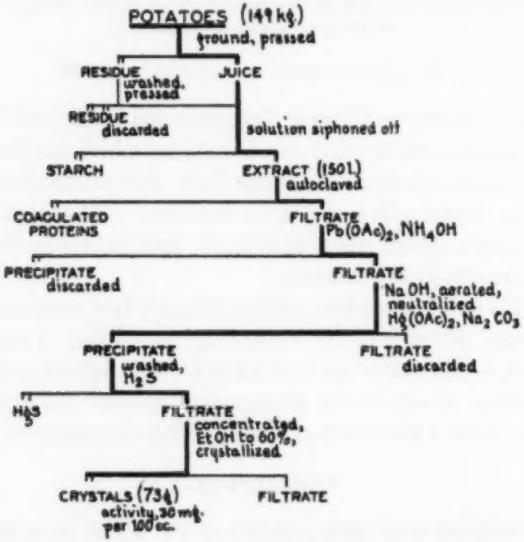

FIG. 1. METHOD OF ISOLATION OF ACTIVE SUBSTANCE

substance which was almost insoluble in 60 per cent alcohol. After washing the crystals with dilute alcohol to remove a gummy material, they were recrystallized from hot dilute alcohol. From 149 kgm. of potatoes 73 grams were obtained. Tests on each discarded fraction showed that the crystalline material alone possessed activity.

Identification. The crystals were proven to be *l*-asparagine by their water of crystallization, total and amid nitrogen content, and specific rotation and by the preparation and analysis of the benzoyl derivative.

A sample was dried over calcium chloride to constant weight, and the water of crystallization driven off at 105°C.

1.2477 grams ($CaCl_2$ dried) lost 0.1522 gram at 105°C.
Calculated for $C_4H_8N_2O_3 \cdot H_2O$ ..... H_2O 12.0
Found ..... H_2O 12.2

Total nitrogen by the Kjeldahl method was run on anhydrous samples (dried at 105°C.).

0.2087 gram gave 31.3 cc. 0.1 N NH_4OH
0.1953 gram gave 29.2 cc. 0.1 N NH_4OH
Calculated for $C_4H_8N_2O_3$ ..... N 21.19
Found ..... N 21.00, 20.94

A determination of amid nitrogen was run on the anhydrous material by measuring the ammonia evolved from boiling 1 N NaOH.

0.5228 gram gave 38.0 cc. 0.1 N NH_4OH
Calculated for $C_4H_8N_2O_3$ ..... N 10.59
Found ..... N 10.18

The specific rotation for a 0.4 per cent solution was $-9.7°$ in water and $+34.7°$ in 10 per cent HCl. The reported values are $-6.2°$ in water and $+37.3°$ in HCl (Mulliken (1918)).

The benzoyl derivative was prepared by dissolving the sample in sodium bicarbonate solution and adding benzoyl chloride with constant shaking (Fischer (1899)). The derivative was precipitated with acid, filtered and washed with alcohol and ether to remove benzoic acid and recrystallized.

0.02396 gram gave 0.04942 gram CO_2, 0.01025 gram H_2O
0.03253 gram gave 0.06710 gram CO_2, 0.01430 gram H_2O
1.0258 gram gave 43.7 cc. 0.1 N NH_2OH (amid nitrogen)

Calculated for $C_{11}H_{12}N_2O_4$	C 55.9	H 5.08	Amid N 5.93
Found	C 56.2	H 4.78	Amid N 5.98
	C 56.3	H 4.93	

The benzoyl derivative had a melting point of 191°—192°C. The same melting point was found for the derivative of an authentic sample of asparagine, and for a mixture of the two preparations.

The properties of the stimulating factor as determined by biological activity during the preliminary investigations were

similar to those of asparagine, namely, solubility in water and insolubility in organic solvents; stability to autoclaving; destruction by ashing; ease of dialysis; non-adsorption; non-precipitation by lead acetate, tungstic acid or phosphotungstic acid; precipitation by Neuberg's reagent; destruction by nitrous acid.

The identification of aspartic acid. *l*-Aspartic acid was isolated by crystallization from the acid hydrolysate of the crystals. The benzoyl derivative was prepared as above and analyzed.

0.02648 gram gave 0.05498 gram CO_2, 0.01142 gram H_2O
0.03084 gram gave 0.06307 gram CO_2, 0.01313 gram H_2O
0.0416 gram gave 9.535 cc. 0.1 N NH_4OH (Kjeldahl)

Calculated for $C_{11}H_{11}NO_5$	C 55.6	H 4.6	N 5.9
Found	C 55.6	H 4.8	N 5.73
	C 55.8	H 4.7	

The insoluble copper salt of aspartic acid was prepared by adding a hot solution of copper acetate to a water solution of the isolated aspartic acid neutralized with sodium hydroxide. On cooling, the copper salt separated out. It was dried at 140°C. and analyzed for copper.

0.1111 gram gave 0.0447 gram CuO

Calculated for $C_4H_5NO_4Cu$	Cu 32.2
Found	Cu 32.7

The specific rotation of the free acid recovered from the copper salt was +22.9° for a 1.25 per cent solution in 10 per cent HCl. The reported values range from +23° to +25° (Mitchell and Hamilton (1929)).

Biological effects

Effect of l-asparagine on the production of butyl alcohol. The stimulating influence of the isolated asparagine is shown in figure 2. With increasing concentration of asparagine the butyl alcohol increased. All samples of commercial asparagine caused a stimulation identical with that brought about by the potato preparation. When the stimulative effect of crude potato extract is plotted against its asparagine content as determined by amid nitrogen, a similar curve is obtained (fig. 2). The slope of the curve is identical with that of pure asparagine and the maximum

is reached at the same concentration. The higher yield is probably due to the presence of aspartic acid or other related compounds which, as will be shown later, also have a stimulatory effect.

Effect of aspartic acid on the production of butyl alcohol. Aspartic acid had an effect quite similar to that of asparagine (fig. 3). Although the maximum yield of butyl alcohol is a little higher than with asparagine, the slope of the curve is not as steep. The

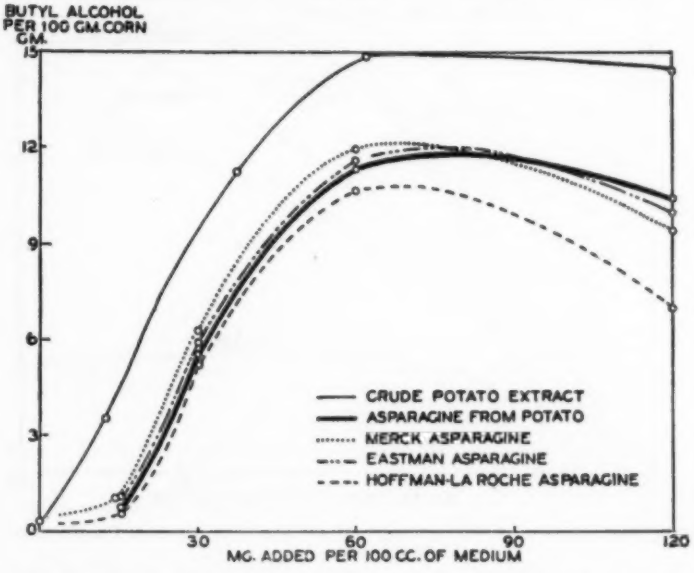

FIG. 2. EFFECT OF ASPARAGINE ON THE PRODUCTION OF BUTYL ALCOHOL

addition of ammonium sulfate in one-half molal quantity per mol aspartic acid overcame this lag without changing the maximum yield of butyl alcohol. The effect of ammonium salts alone was not at all comparable with that of asparagine or aspartic acid.

The activity of the asparagine samples was not due to an adsorbed impurity such as thallium (Richards (1932)), since repeated recrystallization had no effect on their activity. Aspartic acid, recovered from the copper salt, or from the ether-extracted

and recrystallized benzoyl derivative, had the same activity as the original acid. This indicated that the stimulant was aspartic acid itself and not an impurity since it is improbable that an impurity would remain through the formation of a derivative, recrystallization, ether extraction (in the case of the benzoate) and regeneration. The ether extraction was especially conclusive

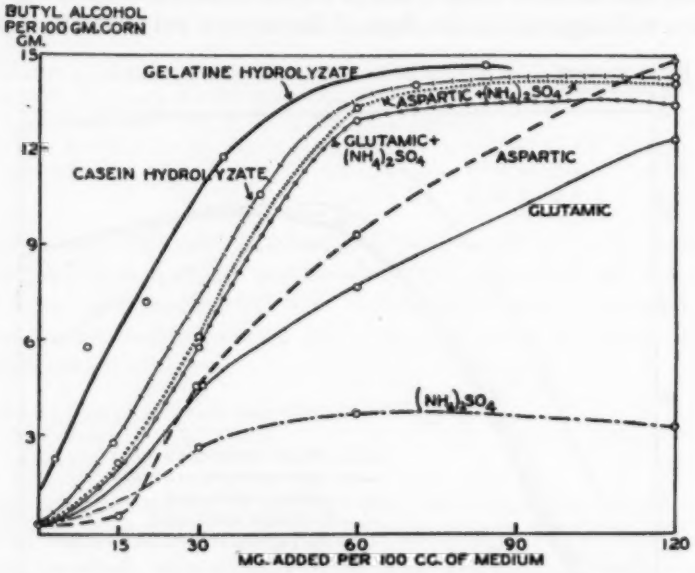

FIG. 3. THE EFFECT OF ASPARTIC AND GLUTAMIC ACID ON THE PRODUCTION OF BUTYL ALCOHOL

Glutamic and aspartic acid neutralized with NaOH before adding to fermentation. Ammonium sulfate added in the ratio of 0.5 mol per mol of amino acid.

since the stimulant as such could under no conditions be extracted with ether.

Effect of other amino acids. A number of other amino acids were tested for stimulatory action, *viz.*, glycine, alanine, leucine, cystine, glutamic acid and a mixture of mono-amino acids obtained from a butyl alcohol extract of casein hydrolyzate (Dakin (1918)). Of these, only glutamic acid had any effect. It increased the

butyl alcohol production just as did aspartic acid, the effect being similarly influenced by the addition of ammonium salts (fig. 3). The specific effect of dibasic amino acids was confirmed by the stimulation caused by casein and gelatin hydrolyzates (fig. 3). Here the production of butyl alcohol was plotted against the amount of dibasic amino acids in the added hydrolyzate. The figure for each protein was calculated from data given by Mitchell and Hamilton[1] (1929). The curves are practically identical with that of aspartic or glutamic acid and ammonium sulfate.

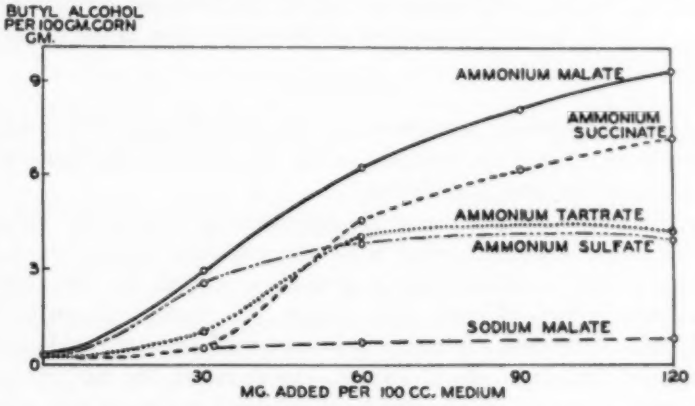

FIG. 4. EFFECT OF AMMONIUM MALATE, SUCCINATE, AND TARTRATE ON THE PRODUCTION OF BUTYL ALCOHOL

Acids neutralized with NH_4OH or NaOH before adding to fermentation

As a result of hydrolysis, there probably was a considerable amount of ammonium sulfate present to increase the stimulative action of the dibasic amino acids. Since casein contains 36.4 per cent dibasic amino acids (including β-OH glutamic acid) while gelatin contains only 9.2 per cent, the remarkable similarity of the curves when calculated on the dibasic acid content is very strong confirmatory evidence that all the dibasic amino acids, and only the dibasic acids, are concerned in the stimulation.

Substitution of related compounds for aspartic acid. Since the stimulative property is apparently confined to asparagine, and the dibasic amino acids, an attempt was made to substitute for

aspartic acid compounds related to it. The ammonium salts of malic, succinic and tartaric acids were used in amounts equivalent to the necessary aspartic acid (fig. 4). Ammonium malate, although not equal to aspartic acid, was the best substitute for it, as would be expected from its structure. Ammonium succinate was found to be fairly good, while ammonium tartrate had no effect beyond that attributable to the ammonium radical itself. This result is not unexpected because tartaric acid is utilized by very few microorganisms, while succinic acid is fairly available. Sodium malate and succinate had no effect on the fermentation probably because of the lack of available nitrogen in the medium.

DISCUSSION

The widespread occurrence of the stimulating substance as previously described is explained by the general distribution of asparagine in natural materials.

The poor fermentation of unsupplemented corn by the butyric acid bacteria as compared with the vigorous fermentation by *Clostridium acetobutylicum* is probably related to the former organism's lack of proteolytic power. *Cl. acetobutylicum* brings about so extensive a proteolysis of corn that glutamic acid can be isolated (Peterson, *et al.* (1924)). In contrast, the butyric acid bacteria bring about no increase in soluble nitrogen in corn either with or without the addition of aspartic acid. The aspartic and glutamic acids required for a vigorous fermentation may be furnished by adding either the pure compounds or a filtrate from a *Cl. acetobutylicum* corn fermentation, or by digesting the corn mash with an enzyme preparation (trypsin) before fermenting.[2]

The stimulative effect of dibasic amino acids and asparagine on the butyric acid bacteria is not surprising since stimulating of bacterial growth has often been associated with these substances. Asparagine has been widely used in growing the tubercle organism in synthetic media. The presence of asparagine may explain the results of Uyei (1930) who reported a growth stimulant for this organism in potato. He showed that the unknown substance

[2] The data on which these statements are based are omitted because they seem somewhat extraneous to the main purpose of this paper.

was insoluble in organic solvents, was not contained in a protein preparation from potato nor in the known carbohydrate constituents.

The rôle of asparagine and similar compounds in the butyric acid fermentation is not certain. In view of the relatively large amounts necessary (60 mgm. per 100 cc. of medium), it is possible that they are essential constituents of the cell protoplasm. The stimulatory effect therefore may be concerned with cell proliferation rather than with metabolism.

The ability of the butyric acid organisms to utilize ammonium malate fairly well and ammonium succinate to a lesser extent in place of asparagine or aspartic acid shows that the structural configuration of the carbon residue and its availability has an effect over and above the nitrogen content alone. This result is similar to that reported for the tubercle bacillus by Henley and Le Duc (1930). They found that ammonium malate was an efficient substitute for asparagine, and that ammonium succinate and tartrate were about one-half as efficient. This substitution might be compared with the substitutions of closely related derivatives for certain essential amino acids in animal nutrition, for example, the substitution of imidazole lactic acid for histidine (Cox and Rose (1926)), and the substitution of 3-indol-pyruvic acid for tryptophane (Berg, Rose and Marvel (1929)).

SUMMARY

The substance in potato extract which stimulates the fermentation of starch and the production of butyl alcohol by certain butyric acid bacteria has been isolated and identified as *l*-asparagine.

l-Aspartic acid and *d*-glutamic acid when supplemented with molal equivalents of ammonium sulfate brought about a stimulative effect equivalent to that of asparagine.

The optimum concentration of these substances was 60 mgm. per 100 cc. of 4 per cent corn-mash, although the effect was noticeable in a concentration of 20 mgm. per 100 cc.

Mono-amino acids and ammonium salts were ineffective in bringing about the stimulation.

Ammonium malate was a good and ammonium succinate a fairly

good substitute for aspartic acid but ammonium tartrate had no effect.

Certain strains of butyric acid bacteria apparently require a four or five carbon dicarboxylic amino acid or a substance which may be transformed into one, in order to ferment starch efficiently and vigorously.

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THE DIFFERENTIATION OF BACILLUS FALLAX (WEINBERG AND SÉGUIN) FROM BACILLUS CARNIS (KLEIN)

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In preceding articles, Duffett (1933) and Hall and Duffett (1935) identified von Hibler's "*Bacillus VI*" (1908) with *Bacillus carnis* (Klein, 1903, 1904) and indicated the differentiation of this organism from *Bacillus fallax* (Weinberg and Séguin, 1915).

B. fallax, first described under the designation "*Bacille A*" in 1915 by Weinberg and Séguin from a fatal case of gaseous gangrene where it was associated with "*V. septique*" and "*B. oedematiens*," was isolated altogether four times from their 125 cases (1916). Since that time, isolation of *B. fallax* has been recorded by Weinberg and Séguin (1917), Henry (1917), Henry and Lacey (1920) from wounds in cases of gaseous gangrene, by Kendall, Day and Walker (1922) from intestinal contents, by Duthie (1924) and Weinberg, Renard and Davesne (1926) from cases of appendicitis, by Mutch and Mutch (1927) from chronic arthritis, and, according to Weinberg and Ginsbourg (1927), by Weinberg and Aynard from a case of blackleg in sheep. In addition, Stokes (1916) obtained anaerobic bacilli, from blood cultures in cases of septicemia following wound infections, which Robertson (1929) identified as *B. fallax*.

It is to be noted that the name "*Bacillus fallax*" as used by Ornstein in 1920 for a Gram-negative, aerobic, intestinal bacterium is invalid on the ground of priority. The writer has examined a culture of Ornstein's "*Bacillus fallax*" and found it to be closely related to, if not identical with, *Bacterium typhosum*.

In 1917 Weinberg and Séguin concluded from the description

of "*Bacillus VI*," as given by von Hibler (1908), that it was impossible definitely to distinguish "*Bacillus VI*" from *B. fallax*, or to identify them. It was the purpose of this brief investigation of *B. fallax* to show that it is a separate species from *B. carnis*.

Since the various reported strains of *B. fallax* were not available, Hall's *B. fallax* No. 254 (American Type Culture Collection No. 3519) was studied as a type strain, as it was one of Weinberg's four original strains.

The following descriptions of morphology and cultural properties of *B. fallax* agree with those given by Weinberg and Séguin (1915, 1917), Henry (1917), Bulloch et al. (1917), Kahn (1922), Kendall, Day and Walker (1922), Duthie (1924), Mutch and Mutch (1927), and Robertson (1929), except where specific exceptions are noted.

MORPHOLOGY

In young cultures the bacilli were Gram-positive, but tended to become Gram-negative in older cultures. The rods were straight or slightly curved and had pointed ends. The bacilli measured 0.6 micron in width by 1.7 to 5.2 microns in length. The average dimensions were 0.6 micron in width by 2.8 microns in length. It may be noted that Duthie found the organisms to be only 0.1 to 0.4 micron in width. Sluggish motility was observed in coverslip preparations of 24-hour liquid cultures. Figure 1, plate 1, shows two bacilli with peritrichous flagella stained by Bailey's (1930) method in a mount from a 22-hour culture on blood agar slant under alkaline pyrogallol. Morphologically, *B. fallax* was very similar to *B. carnis*, except that *B. carnis* had rounded ends.

Spore formation occurred only in mediums not containing an excess of fermentable carbohydrates. Mature refractile spores were seldom seen even in old cultures, but solidly staining orgonts were observed regularly in small numbers on blood agar slants under alkaline pyrogallol, when stained with the ordinary aniline dyes after 48 hours incubation at 37°C. They were usually subterminal, but frequently central and occasionally terminal, and always much larger in diameter than the rods.

Figure 2, plate 1, shows vegetative rods and orgonts of *B. fallax*, from such a culture, solidly stained by Gram's method.

Spores of *B. carnis* were produced in greater numbers under identical conditions. They were usually refractile, were nearer the ends of the rods, and swelled the rods less. Cultures of both species were boiled for 2 minutes at 94°C. without destruction of the spores. Weinberg and Séguin found spores in only one of their four strains of *B. fallax*, but recorded no observations as to their resistance to heat.

CULTURAL PROPERTIES

Upon incubation of this organism at 37°C., growth in various anaerobic mediums and devices used in this laboratory (Hall, 1932) appeared in from 24 to 48 hours, as it did with *B. carnis*. Visible growth of both species failed to appear at 20°C. until after three or more days. In contrast to *B. carnis*, *B. fallax* failed to grow aerobically on any medium; *B. fallax* is an obligate anaerobic.

Deep brain medium containing iron wire. Turbidity and gas were produced in 24 hours incubation. There was no blackening or production of tyrosine crystals in 60 days. In these respects there was no difference between *B. fallax* and *B. carnis*. Henry (1917), however, reported the blackening of a meat medium by *B. fallax*.

Glucose broth in constricted tubes with marble seals. Turbidity, acid, and gas were produced by both species on 24 hours incubation. Many of the cells had settled to the bottom of the tube after 48 hours.

Gelatin in constricted tubes. Turbidity and gas were produced by both species in 24 hours, without liquefaction of the medium even in 30 days.

Blood agar under alkaline pyrogallol. Dew-like colonies of both species developed in 24 hours. The colonies of *B. fallax* increased in size to 1 to 1.5 mm. in diameter in 48 hours, and became slightly raised and irregular in shape. On further incubation, the colonies became opaque. The *B. fallax* colonies were found to be unlike those of *B. carnis* in that they were non-

hemolytic and never tended to round up into pearly beads or to become confluent. Figure 3, plate 1, shows colonies of *B. fallax* on a blood agar slant under alkaline pyrogallol after 96 hours incubation. On 1 per cent glucose blood agar the colonies were similar to those on plain blood agar. They remained nonhemolytic.

Milk in constricted tubes. Both species produced gas in 24 hours, but *B. fallax*, in contrast with *B. carnis*, definitely coagulated the casein in about 7 days; neither digested the casein within 30 days.

Deep 1 per cent agar. Minute lenticular colonies appeared with both species in 24 hours. There was some production of gas. *B. fallax* never showed outgrowths such as were seen with *B. carnis*, and the colonies were always much smaller and more compact. I have not seen the "coeurs jaunes" colonies described and illustrated by Weinberg and Séguin (1917). Figures 4 and 5, plate 1, show 8-day-old colonies in deep methylene blue (1:100,000) agar. Figure 6 shows a similar colony of *B. carnis* for comparison. This particular colony was taken from agar containing no methylene blue. The dye had no effect upon the size and shape of the colonies.

Difco "Bacto-tryptone." No indol was detected with either species in 24-to 96-hour cultures in 1 per cent solutions of "Bacto-tryptone" in distilled water in constricted tubes by the Ehrlich-Böhme and Goré tests as recommended by Conn et al. (1932).

Fermentative reactions. There is general agreement that *B. fallax* ferments glucose, galactose, levulose, mannose, maltose, sucrose, raffinose, and xylose and that it does not ferment dulcitol, sorbitol, glycerol and inositol. Both fermentation and nonfermentation of lactose, arabinose, salicin, mannitol, dextrin, starch, and inulin have been reported. Kahn (1922) found that rhamnose and amygdalin were not fermented.

My attempts to determine the fermentative reactions of *B. fallax* showed that glucose, galactose, levulose, mannose, maltose, lactose, sucrose, raffinose, xylose, rhamnose, arabinose, salicin, amygdalin, mannitol, and dextrin were fermented, and that starch, inulin, dulcitol, sorbitol, glycerol, and inositol were not fermented.

Discordant results in the reports of the various workers are probably due to the difficulty of obtaining growth in the mediums used, and to the possibility that the various strains were not identical with the type strain described by Weinberg and Séguin and studied by the author.

It is to be noted that *B. fallax* fermented raffinose, xylose, rhamnose, mannitol, and arabinose, in addition to the carbohydrates fermented by *B. carnis*.

PATHOGENICITY

According to Weinberg and Séguin (1915, 1917), some recently isolated strains of *B. fallax* were pathogenic for guinea pigs, but this property disappeared upon artificial incubation. They also found that a soluble toxin was produced. Duthie (1924) reported that his strains were weakly pathogenic. Mutch and Mutch (1927) observed their strain to be nonpathogenic for mice and guinea pigs. However, they reported improvement of arthritic patients when *B. fallax* was given as a vaccine.

The type strain studied here has shown no evidence of pathogenicity in contrast to *B. carnis* which has remained pathogenic 36 years after isolation Subcutaneous injection of one cubic centimeter of a 24-hour glucose broth culture of *B. fallax* failed to produce any visible effects in guinea pigs weighing from 250 to 300 grams. Intravenous injection of one cubic centimeter of a similar culture was nonpathogenic for a rabbit weighing 1550 grams No pathogenicity was encountered in the preparation of an agglutinating serum in a rabbit weighing 1750 grams, in contrast to the great difficulty encountered in preparing antiserums with *B. carnis*.

SEROLOGY

Agglutinating serums for *B. fallax* were prepared by Weinberg and Séguin (1915, 1917), Duthie (1924), and Mutch and Mutch (1927). These serums agglutinated the homologous strains, but action on other strains was inconstant.

In order to show serologic differences between *B. fallax* and *B. carnis*, anti-*B. fallax* agglutinating serum was prepared in a white

rabbit weighing 1750 grams by the injection of a total of 19 cc. of 2- to 3-day-old glucose-broth cultures over a period of 38 days. No agglutinins for *B. fallax* were demonstrable in a 1:20 dilution of the serum of the rabbit before the injections The initial dose of 1 cc. was made intravenously. The remainder of the injections, 2 cc. each, were made subcutaneously. The rabbit was bled ten days after the last injection and serum was obtained for agglutination tests.

The serum agglutinated *B. fallax* in all dilutions up to 1:1280. It did not agglutinate any of the four strains of *B. carnis* at 1:20; nor did 1 cc. of the serum protect against 0.05 cc. of a 24-hour glucose broth culture of any strain of *B. carnis* Hall and Duffett (1935) reported cross-agglutination between all of the four strains of *B. carnis*. Serums used in those tests failed to agglutinate *B. fallax* at 1:20 or higher.

SUMMARY

Morphological, cultural, biochemical, pathogenic and serological differences between *B. fallax* and *B. carnis* have been described.

B. fallax and *B. carnis* are separate and distinct species of bacteria.

The author is indebted to Dr. Ivan C. Hall for cultures and suggestions, to Miss Elizabeth O'Toole for the flagellar stain, and to Mr. Glen Mills for the photographs.

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PLATE 1

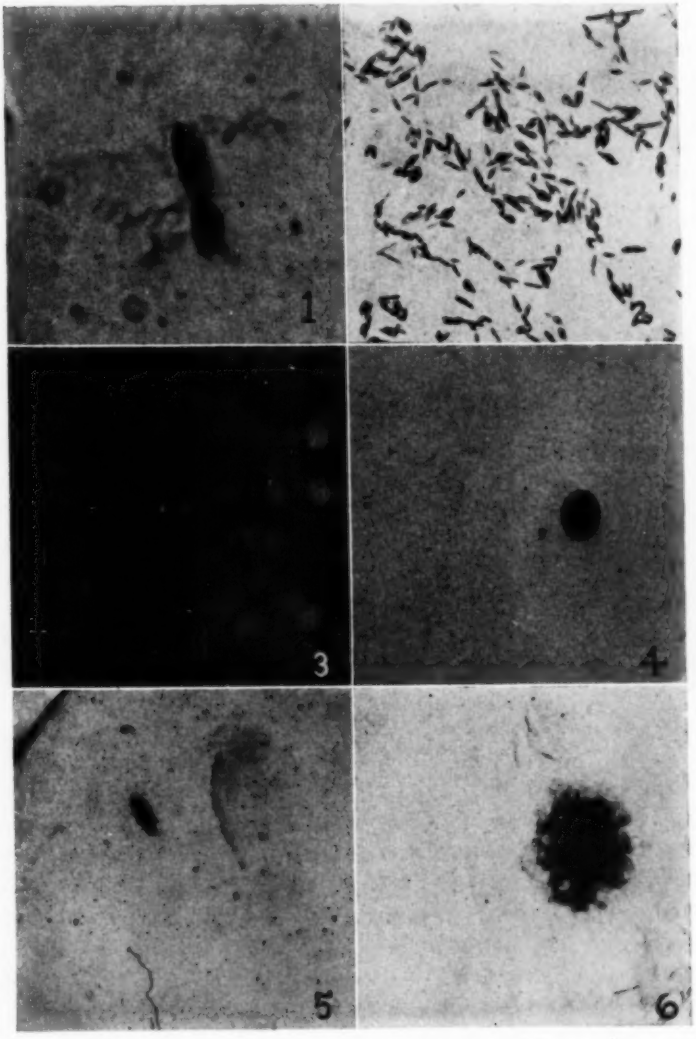
FIG. 1. *B. fallax* with flagella stained by Bailey's method from a 22-hour culture on blood agar slant under alkaline pyrogallol. × 3300.

FIG. 2. Vegetative rods and orgonts of *B. fallax* stained by Gram's method from a 48-hour culture on blood agar slant under alkaline pyrogallol. × 1000.

FIG. 3. Colonies of *B. fallax* on blood agar slant under alkaline pyrogallol after 96 hours incubation. × 6.

FIGS. 4 AND 5. Colonies from 8-day culture of *B. fallax* in deep methylene blue 1 per cent agar. × 10. Figure 5 shows a colony viewed edgewise.

FIG. 6. Colony from 8-day culture of *B. carnis* in deep 1 per cent agar. × 10.



(Nicholas D. Duffett: Differentiation of B. Fallax and B. Carnis)

STUDIES IN MICROBIC DISSOCIATION

II. CHANGES IN THE BIOLOGICAL CHARACTERS OF SHIGELLA PARADYSENTERIAE DURING DISSOCIATION AND PARTIAL REVERSION

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The original smooth culture of *Shigella paradysenteriae* Flexner from which rough colony and smooth colony variants were obtained for this study was isolated in this laboratory in 1927 from the stool of a patient during an outbreak of summer diarrhea. Its antigenic relationships and biological characters have been described in a previous communication (Mackenzie and Batt, 1930). Between 1927 and 1932 this culture, 351O, was maintained on sealed agar slants in the ice chest, with subcultures made every three months. During this period it was repeatedly plated and cultures from single colonies were examined serologically and biochemically. There was never any evidence of dissociation. In colonial morphology, type of growth in fluid media, agglutinative and absorptive capacities, and biochemical reactions, the results were uniform. On April 30, 1932, it was plated; a culture from an isolated colony on one of these plates was used throughout the experiments to be described; from another colony on the same plate a tube of beef-infusion formalin broth was seeded. After 26 days' growth in beef-infusion formalin broth, rough colonies were obtained. At the outset the concentration of formalin in the beef-infusion broth was 1:8000. As the culture became adapted to the formalin medium the concentration of formalin was gradually increased. Daily seedings of agar plates from the formalin broth cultures were made, and every 5 days a subculture in fresh medium. When, after 26 days, rough colonies first appeared on the plates, repeated

picking and reseeding from rough colonies soon yielded a stable rough colony variant (351 R) which on agar plates produced 100 per cent rough colonies. The rough colony variant obtained in this way was then subjected to three different types of stimulus to reversion: (1) Daily transfers in beef-infusion broth; (2) growth in 2 per cent homologous immune-serum broth; (3) growth in beef-infusion broth to which had been added 2 per cent vaccine prepared by heating a suspension of the original smooth culture at 60°C. for one hour. From platings of these cultures which had been exposed to reversion stimuli, 4 cultures (351 V, 351 RHIS, 351 L, 351 Q) were selected for study and comparison with the original smooth culture (351 O) and with the rough variant (351 R). The 4 reversion cultures were obtained as follows:

351 V was grown from a smooth colony which appeared on a plate after 20 subcultures in vaccine broth over a period of 65 days.

351 RHIS was obtained after 7 subcultures in 2 per cent homologous immune-serum broth over a period of 35 days; in colonial morphology it had the typical rough appearance;

351 L was obtained after 104 daily subcultures in beef-infusion broth; its colonies were smooth, round, glistening—indistinguishable morphologically from the original smooth culture (351 O);

351 Q was obtained from the culture grown in homologous immune-serum broth; it was grown from a colony picked from a plating after 12 transfers over a period of 62 days; its colony has a round margin, is dome-shaped, but, when examined under a dissecting microscope with reflected light, constantly shows a slight roughening of the surface. Its colony is, however, essentially smooth.

The methods used in the agglutination and absorption procedures and in the preparation of sera have been described in a previous paper (Mackenzie and Fitzgerald, 1933). Indol production was determined by the vanillin test.

The sequence of events in the dissociative and revertive processes just described is shown schematically in chart 1.

In table 1 data on the fermentations and indol production of the original smooth culture, its rough colony variant and the 4 reversion cultures are presented. None of the 5 variants is quite like the parent culture. All of the variants have lost the capacity to

ferment mannitol. Otherwise the fermentations of the variants do not differ from those of the original culture; none has acquired the capacity to form gas from any of the carbohydrates used. All of the variants form indol; the parent culture does not. Thus,

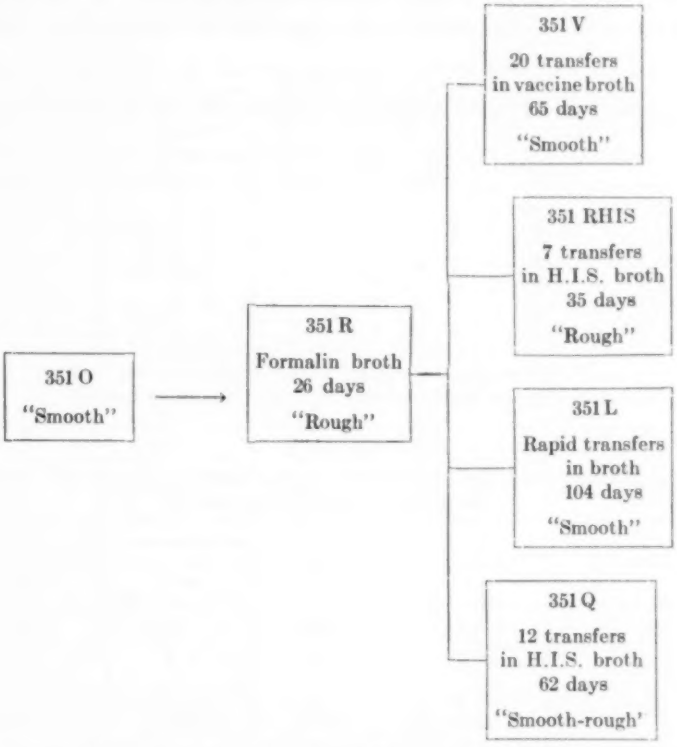

CHART 1. SCHEMA OF DISSOCIATION AND PARTIAL REVERSION OF SHIGELLA PARADYSENTERIAE FLEXNER

it is evident that the dissociative process, growth in formalin broth, has caused the loss of one biochemical function and the acquisition of another; the environmental conditions of the revertive processes have not modified any of the biochemical functions upon which observations were made.

ANTIGENIC RELATIONSHIPS OF THE SMOOTH PARENT CULTURE AND ITS ROUGH VARIANT

In table 2 the results of the reciprocal absorptions of the original smooth culture (351 O) and its rough colony variant (351 R) are shown. 351 R is quite inagglutinable in the 351 O serum and absorbs none of the homologous agglutinins. 351 O

TABLE 1

Fermentations of Shigella paradysenteriae Flexner and variants derived from it

CULTURE	TYPE OF COLONY	MALTOSE	GALACTOSE	DULCITOL	LEVULOSE	GLUCOSE	MANNITOL	INDOL
351 O	(S)	?	+	−	+	+	+	−
351 R	(R)	?	+	−	+	+	−	+
351 RHIS	(R)	−	+	−	+	+	−	+
351 V	(S)	?	+	−	+	+	−	+
351 L	(S)	?	+	−	+	+	−	+
351 Q	(S)	−	+	?	+	+	−	+

− = no acid and no gas; + = acid; ? = very slight transitory acidity.
None of the cultures fermented sucrose, xylose, lactose or dextrin.

TABLE 2

Antigenic relationships of the original smooth Shigella paradysenteriae culture (351 O) and its rough variant (351 R)

SERUM	CULTURE	TITER	AGGLUTINATION AFTER ABSORPTION BY	
			351 O	351 R
351 O	351 O	2560	0	2560
	351 R	0	0	0
351 R	351 O	80	0	80
	351 R	2560	2560	0

agglutinates only to a very low titer in the rough culture serum and fails to absorb any of the homologous agglutinins from this serum. The 351 R culture is completely incapable of absorbing from the homologous serum the heterologous agglutinins for the smooth 351 O culture. This is another example of a culture which causes the formation of agglutinins which it is unable to absorb. Stated in another way, this demonstrates an absence of

parallelism between agglutinogenesis, on the one hand, and agglutination and absorptive capacity on the other. Dissociation appears to have caused a change in antigenic structure which is characterized by loss of capacity to bind the smooth agglutinins but with retention of a limited capacity to cause, in the immunized

TABLE 3

Antigenic relationships of the rough variant (351 R) of Shigella paradysenteriae (351 O) and four reversion cultures (351 RHIS, 351 V, 351 Q, 351 L) derived from it

SERUM	CULTURE	TYPE OF COLONY	TITER	AGGLUTINATION AFTER ABSORPTION BY	
				351 R	351 RHIS
351 R	351 R	(R)	2560	0	0
	351 RHIS	(R)	2560	0	0
351 RHIS	351 R	(R)	2560	0	0
	351 RHIS	(R)	2560	1280	0
				351 R	351 V
351 R	351 R	(R)	2560	0	0
	351 V	(S)	2560	0	0
351 V	351 R	(R)	1280	0	0
	351 V	(S)	1280	0	0
				351 R	351 Q
351 R	351 R	(R)	2560	0	1280
	351 Q	(S)	80	0	0
				351 R	351 L
351 R	351 R	(R)	2560	0	0
	351 L	(S)	1280	0	0

rabbit, the formation of agglutinins reacting with the smooth antigens.

ANTIGENIC RELATIONSHIPS OF THE ROUGH COLONY VARIANT AND THE REVERSION CULTURES DERIVED FROM IT

The reciprocal absorptions of 351 R and 351 RHIS, shown in table 3, reveal identical agglutinogenetic and agglutinative

capacities but a difference in absorptive capacity. Each culture removes all agglutinins from its homologous serum. But 351 R, even though it agglutinates to titer in 351 RHIS serum, is capable of absorbing from this serum only 50 per cent of the agglutinins for 351 RHIS. Here there is a lack of parallelism between agglutinogenesis and agglutination, on the one hand, and absorptive capacity on the other.

The reciprocal reactions of 351 R and 351 V afford an example of complete antigenic similarity. It is significant that these 2 cultures, although antigenically indistinguishable, are very different in colonial morphology. They are also dissimilar in cellular morphology, saline stability, type of growth in fluid media, and zone of acid agglutination. The reversion environment has, therefore, caused an R → S change of several biological characters without altering the antigenic structure. The complete similarity in antigenic behavior of 351 R and 351 V extends to their agglutinative and absorptive capacities in the heterologous sera of this series.

Since no sera have been prepared with 351 Q and 351 L, the data for a comparison of the antigens of these 2 cultures and those of 351 R are incomplete. It is obvious that 351 Q and 351 R have very different antigenic properties. The former agglutinates only to 80 in 351 R serum and absorbs only 50 per cent of the homologous agglutinins from this serum The 351 L culture, as far as the data go, appears to be very similar to 351 R. Their agglutination titers in 351 R serum show a difference of only one tube and their capacities to absorb agglutinins from the 351 R serum are identical. In colonial morphology, saline stability and growth in fluid media, 351 L is similar to 351 O. In fermentations and indol production it is similar to 351 R. Hence, in the reversion process some characters have reverted and others have remained similar to those of the rough variant, 351 R.

ANTIGENIC RELATIONSHIPS OF THE SMOOTH PARENT CULTURE AND THE FOUR REVERSION CULTURES

In table 4 data which make evident the antigenic dissimilarity of the 4 reversion cultures and the original smooth parent culture

are presented. It is quite obvious from this table that all 3 of the antigenic properties of 351 RHIS and 351 V are different from those of 351 O, and that the agglutinability and absorptive capacities of 351 Q and 351 L are different from those of 351 O. Thus, none of the reversion cultures reverted in antigenic struc-

TABLE 4

Antigenic relationships of the original Shigella paradysenteriae culture (351 O) and four reversion cultures (351 RHIS, 351 V, 351 Q, 351 L) derived from its rough variant (351 R)

SERUM	CULTURE	TYPE OF COLONY	TITER	AGGLUTINATION AFTER ABSORPTION BY	
				351 O	351 RHIS
351 O	351 O	(S)	2560	0	2560
	351 RHIS	(R)	0	0	0
351 RHIS	351 O	(S)	80	0	80
	351 RHIS	(R)	2560	2560	0
				351 O	351 V
351 O	351 O	(S)	2560	0	2560
	351 V	(S)	0	0	0
351 V	351 O	(S)	160	0	0
	351 V	(S)	1280	320	0
				351 O	351 Q
351 O	351 O	(S)	2560	0	2560
	351 Q	(S)	0	0	0
				351 O	351 L
351 O	351 O	(S)	2560	0	2560
	351 L	(S)	0	0	0

ture to even approximate identity with the original smooth culture from which it was indirectly derived.

INTERRELATIONSHIPS OF BIOLOGICAL CHARACTERS OTHER THAN ANTIGENIC STRUCTURE

A summarized tabulation of the morphological and physiological characters upon which observations were made is shown

TABLE 5

Summary of biological characters of the parent smooth culture of Shigella paradysenteriae (351 O), its rough colony variant (351 R) and the four reversion cultures (351 RHIS, 351 V, 351 L, 351 Q)

	351 O	351 R	351 RHIS	351 V	351 L	351 Q
Colonial morphology	Smooth (at room temperature mucoid)	Rough	Rough	Smooth	Smooth	Smooth
Cellular morphology	Small Gram-negative rods. Very slight pleomorphism	Slender Gram-negative rods. Longer and more pleomorphic than 351 O. A few filamentous forms	Same as 351 R	Same as 351 O	Similar to 351 O but has occasional long forms	Similar to 351 L
Stability in NaCl	Stable	Flocculates in 0.6 per cent NaCl	Flocculates in 0.6 per cent NaCl	Stable	Stable	Stable
Growth in fluid media	Dispersed	Very slightly floccular	Floccular, pellicle ++, sediment ++	Dispersed	Dispersed	Floccular, pellicle ++, sediment ++
Acid agglutination zones (citrate buffer-Clark)	pH 1.1 to 1.9	pH 1.1 to 4.8	pH 1.1 to 4.9+	pH 1.1 to 3.5	pH 1.1 to 3.5	pH 1.1 to 4.6

Fermentations	Glucose, levulose, galactose, mannitol	Glucose, levulose, galactose	Glucose, levulose, galactose	Glucose, levulose, galactose	Glucose, levulose, galactose	Glucose, levulose, galactose
Indol	Negative	Positive	Positive	Positive	Positive	Positive

in table 5. Study of this table fails to reveal evidence of firm linkage between any two of the biological characters of these cultures, except perhaps between rough colony form and instability of suspensions in NaCl. There is, to be sure, an apparent correlation between loss of the capacity to ferment mannitol and ability to form indol, but from other evidence it is clear that no linkage exists between mannitol fermentation and inability to form indol.

DISCUSSION

The results of this attempt to define and analyze the alterations of the biological characters of a smooth colony culture of *Shigella paradysenteriae* make possible certain deductions regarding two aspects of dissociation. The deductions cannot, of course, be applied to the phenomena of dissociation in cultures of other genera and perhaps only tentatively to other species of the same genus.

First, it is possible to state that under the conditions of these experiments none of the variant cultures has reverted completely to the morphological, physiological and serological status of the original smooth parent culture. Two or more of the biological characters have been shown to have reverted, but when the analysis is carried to other biological characters differences between parent culture and reversion culture stand out conspicuously. Reversion has in no instance been complete. The methods used to bring about reversion are those which, in the present state of knowledge of the subject, would seem most likely to cause complete reversion if complete reversion of a well stabilized colonially and antigenically completely rough Shigella variant is indeed possible. From published observations (Schütze, 1921; Arkwright and Pilt, 1929; Savage and White, 1923–5) and our own efforts to cause the R → S change of this and other cultures we, at the present time, are inclined to believe that complete reversion rarely, or perhaps never, occurs in the Shigella and Salmonella genera once the variant has been shown to be stabilized in a state of extreme roughness. Contrasting with this irreversibility in these genera, the work of Griffith (1928), Dawson

(1930), and Dawson and Sia (1931) with *Diplococcus pneumoniae* has shown reversion, apparently complete, of avirulent rough cultures.

The second aspect of dissociation upon which our observations have a bearing is the nature of the demonstrable changes which take place in a dissociating culture. While the results reported in this paper by no means justify any conclusions regarding the mechanism of the profound alterations of the biological characters of a culture occurring during dissociation, they provide data which are pertinent to this obscure question. If one biological character of a culture may, as a result of altered environmental conditions, undergo a change without any demonstrable alteration in other biological characters, we have data which are useful in formulating an hypothesis regarding the nature of dissociation. If the biological characters of a culture are capable of independent variation, the cyclostage theory of dissociation is less tenable.

Viewed as a whole, the alterations of the biological characters of the original smooth *Shigella paradysenteriae* culture during the dissociative and revertive processes have occurred without evidence of unalterable linkage. With the possible exception of rough colony form and saline instability, no two characters have been observed to change in a hand-in-hand fashion. The smooth colony form of the original culture was found to occur in variants with altered biochemical functions. So, too, the same colony form was observed in variants differing widely in antigenic functions. 351 V and 351 L, although producing colonies indistinguishable from those of the original smooth culture, are very different from it in agglutinative, absorptive and agglutinogenic properties. Furthermore, the data tabulated in tables 3 and 4 show an independent variability of these three functions of an antigen. Absorptive capacity may change while agglutinability and agglutinogenesis remain unaltered (cf. 351 R and 351 RHIS); or agglutinability and absorptive capacity may be so modified that they yield no hint of kinship between a parent culture and its variant, while agglutinogenesis still demonstrates a common antigen (cf. 351 O and 351 R).

CONCLUSIONS

1. By growing a smooth culture of *Shigella paradysenteriae* Flexner in formalin broth, a stabilized variant yielding constantly 100 per cent rough colonies was obtained. The serological, morphological and physiological characters of this rough variant differed from those of the parent culture.

2. When the stabilized rough colony variant was subjected to environmental conditions (growth in homologous immune serum, growth in beef-infusion vaccine broth, and rapid transfers in plain broth) which favor reversion to the normal smooth form, it yielded four reversion cultures, no two of which were alike.

3. All of the reversion cultures differed in one or more biological character from the rough variant and from the original smooth culture. Reversion, therefore, was in no instance complete.

4. The agglutinative, agglutinogenic and absorptive capacities of *Shigella paradysenteriae* Flexner may, as a result of dissociation, vary independently of each other.

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BACTERIAL GROWTH WITH AUTOMATIC pH CONTROL

(A) AN APPARATUS. (B) SOME TESTS ON THE ACID PRODUCTION OF LACTOBACILLUS ACIDOPHILUS

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Since much modern bacteriological work requires large quantities of bacteria, and an important limiting factor in the growth of acid-forming bacteria is the decrease of the pH of the media, it has been considered desirable to design and test a device which will keep the pH constant at any chosen value during bacterial growth. Experiments on *Lactobacillus acidophilus* with the aid of this apparatus have yielded data on the rate of acid production at various pH values, and on the effect of dissolved gases on the bacterial growth. The following paper contains a description of the apparatus, and an account of some typical experiments.

A. THE APPARATUS

The device about to be described maintains a constant pH value by adding automatically at intervals the necessary amount of a solution of an alkali.[1] Glass electrodes are used for the measurement and control of the pH, since other types of electrodes require conditions and reagents not suitable in bacteriological media, and are rendered inaccurate, i.e., "poisoned," by such media. Glass electrodes, on the other hand, have no effect on the solutions in which they are placed, and their potentials respond, within their range of usefulness (about pH 1 to pH 9), only to the pH. The apparatus as a whole is shown diagrammatically in figure 1. The solution, the pH of which is to be controlled, is placed in the flask

[1] An apparatus for a similar purpose, but differing widely from ours in details, has been described by Whitnah, C. H., Ind. Eng. Chem. Anal. Ed., **5**, 352 (1933).

F. The pH is measured by the potential of a galvanic cell consisting of the glass electrode *G* and the reference calomel electrode *C*, both connecting with the solution in *F*. The glass electrode used is of the "durable" spiral type described by MacInnes and Belcher (1933). The calomel electrode, which is made with 3.5 N KCl, connects with the solution in flask *F* by means of a column of potassium chloride solution of the same concentration

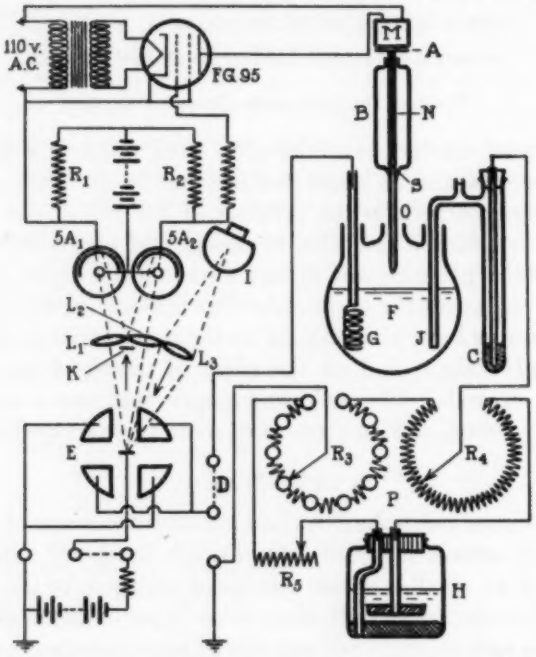

FIG. 1. DIAGRAM OF THE APPARATUS FOR MAINTAINING pH VALUES

and terminates in a very dense porous plug at the point *J*. The electromotive force of this galvanic cell is opposed by a known potential from the potentiometer *P*, and a condition of balance is indicated by the Compton electrometer *E*.

The circuit which controls the addition of alkali is arranged as follows. The light from a 32-candle power automobile headlight bulb *I* is focussed on the electrometer mirror with a lens

L_3. The reflected beam is divided between two lenses, L_1 and L_2, and is collected by two Western Electric vacuum phototubes, $5A_1$ and $5A_2$. These phototubes are placed in the opposite arms of a Wheatstone bridge. The bridge is completed by means of two fixed resistances, R_1 and R_2, which have values (10 to 12 megohms) comparable to the resistances of the illuminated phototubes. A 45-volt "B" battery supplies the bridge potential. The bridge terminals, normally joined to a galvanometer or other null point indicator, are connected, instead, to the control grid and filament of a double grid "thyratron" FG 95. The thyratron plate circuit includes the electromagnet M, which controls the flow of alkali from the buret B to the solution in the flask F.

The resistances, R_1 and R_2, are selected so that the thyratron grid potential is near the critical value when the phototubes have approximately equal illumination. A fine adjustment for bringing the grid bias very close to its critical value is obtained by shifting a small opaque screen at K. This screen may also be used to compensate for drifts in the natural zero of the electrometer needle.

A diagram of the magnetically operated buret is also included in figure 1. When current is passing, the magnet armature A lifts a glass rod, N, which extends the full length of the buret and carries a small rubber plug. The capillary outlet, O, is sealed through the lower end of the buret with a projecting shoulder, S, against which the rubber plug normally presses. When the plug is lifted from the shoulder the liquid flows, under gravity, from the buret. Since the magnet armature moves only 1 mm. in opening and closing the valve, a sufficiently strong pull can be obtained with a small current through the magnet. This magnet is also equipped with a small oil dashpot for the elimination of chattering. The valve avoids the lubrication necessary with ordinary stopcocks, a decided advantage in dealing with strong alkali, as in this work. The rate of flow from the buret may be adjusted by varying the length and bore of the capillary outlet.

The flask F is shown in more detail in figure 2. Alkali is added through the tube at U. The medium is agitated by the stirrer Z. A water seal for the stirrer is provided at W. Inoculation of the

medium and withdrawal of samples for study are carried out through tube X which is normally plugged with cotton wool. Gas can be bubbled through the medium by means of the tube Q–T and can escape through the cotton plug. The reference electrode dips into the upper part of the tube C'–J. Contamination by air-carried bacteria is minimized by making the ground glass joints of the skirted type shown in the figure. The flask,

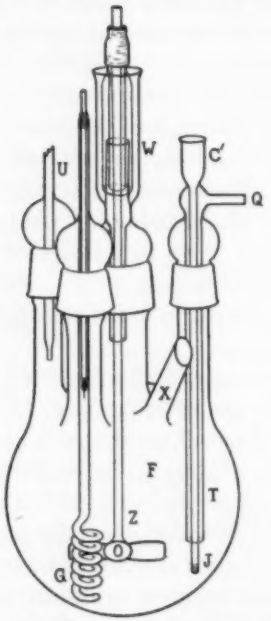

FIG. 2. SPECIAL FLASK FOR GROWING BACTERIAL CULTURES WITH pH CONTROL

and the medium in which the bacteria are grown, are sterilized in an autoclave.[2] Since, however, the glass electrode disintegrates rapidly and loses its calibration in steam or hot water, it is given a

[2] It was not considered necessary to autoclave the buret and its contents since it always contained a solution of sodium hydroxide at a concentration of at least 2 normal. The buret outlet was thoroughly flamed before connecting it to the flask F after the latter had been sterilized in the autoclave. If, however, it should be desirable to use, in the buret, solutions that need sterilization there is no reason why the buret and flask could not go into the autoclave as a unit.

chemical sterilization with 0.1 per cent mercuric chloride and is then rinsed several times, out of contact with laboratory air, in sterile water, before introduction into the flask. A direct test indicated that the mercuric chloride, in amounts sufficient to affect bacterial growth, is removed after two rinsings. Trials are now being made of an apparatus by means of which the chemical sterilization and rinsing of the glass electrode, and its insertion into the flask *F*, may be carried out with no possible contamination by air-borne organisms.

The operation of the apparatus is as follows. After calibration of the glass electrode and the calomel half cell with appropriate buffers, the potentiometer may be set at a value corresponding to the pH at which the system is to be maintained. Assuming that the potentials are now exactly balanced, the electrometer needle will be in its zero position and the light beam reflected from the mirror attached to this needle will be divided between the two phototubes in such a ratio that the thyratron is on the verge of operation. As acid is produced in flask *F* of figure 1 the pH of the solution drops and the electromotive force of the system is no longer balanced by that of the potentiometer. The result is that the electrometer needle rotates slightly from its zero position, thus causing a decrease in the light flux to one phototube and a corresponding increase to the other. The resulting shift in the balance of the Wheatstone bridge causes the thyratron to operate and the opening of the magnetic valve allows alkali to flow into the solution from the buret until a sufficient quantity has been added to restore the pH of the system to its preassigned value. This cycle of operations is repeated automatically and as often as necessary until the experiment is terminated.

With the present arrangement a change of 1 mv. in the potential of the glass electrode causes the thyratron to operate. This corresponds to about 0.02 of a pH unit. In actual operation, however, the deviations of the pH from a constant value are approximately ± 0.05 of a pH unit. Possible causes of deviations occurring while the instrument operates over long periods of time are as follows: (1) Fluctuations of the working current through

the potentiometer. (2) Variations in the intensity of the light source. (3) Shifts in the natural zero of the electrometer needle. (4) Variations of the glass electrode. (5) Variations of the liquid junction potential. These will be discussed in the order given.

1. Variations of the potentiometer current. The usual method for reducing errors from this source is to adjust the potentiometer frequently against a standard cell. However, if this method were adopted the apparatus would not be automatic. The necessity for such adjustments has been avoided by the use of a high resistance potentiometer and a Hulett (1903) standard cell, which can deliver a small current without measurably affecting its potential of 1.019 volts. The potentiometer consists of a decade unit, R_3 of figure 1, with 10,000 ohm steps and a 10,000 ohm variable unit, R_4, which is divided into 100 parts of 100 ohms each. A small supplementary unit, R_5, permits adjustment of the total resistance so that the drop across each division of R_4 is 1 mv. and that across each step of R_3 is 100 mv. The potentiometer is thus graduated in millivolts and can be read somewhat more closely than this, although the present application does not warrant the greater accuracy.

The Hulett standard cell, H, which supplies the potentiometer current is essentially a heavy-duty unsaturated Weston type standard cell. It is, however, designed to have a very low internal resistance and with the additional feature that electromotive force changes due to concentration polarization are largely eliminated. Tests have shown that the potential of this cell remains constant to better than 1 mv., even when furnishing current for the potentiometer, for an interval of several months. Within the accuracy desired errors due to variations in the working current of the potentiometer have thus been entirely eliminated.

2. Variations in the light source. The light source for the phototube bridge is operated from the 110-volt line through a step-down transformer. The fluctuations in the light are mainly due, therefore, to variations in the line voltage. The use of two phototubes in the bridge circuit, as has been described, largely eliminates the errors from this source since, when equally illumi-

nated, a variation in the light source affects the two tubes nearly equally and does not shift the bridge balance appreciably.

3. The zero shift of the electrometer. The apparatus will, obviously, regulate at the desired pH value only so long as the natural zero point of the electrometer does not shift. It has been our experience that the main, if not the only, cause of such shifts is slight variation in the level of the instrument. Such variations have been largely overcome by mounting the electrometer and the light system on a sturdy bracket attached to the outside brick wall of the laboratory. Any zero shifts, and changes in the operating characteristics of the thyratron, can be discovered by reversing the position of the double throw switch *D*, thus grounding the quadrants, and noting whether an adjustment of the screen *K* is necessary to cause the thyratron and magnet *M* to operate. This is the only adjustment found necessary on the apparatus and is made twice a day. If neglected during a run of 100 hours it may result in an error of the order of 0.1 pH unit.

4. Variations of the glass electrode. It is also clear that the apparatus will maintain a constant pH value only if the glass electrode maintains its calibration throughout the period of use. Although the spiral type of electrode has large "asymmetry potentials" for which allowance must be made, these potentials attain steady values after the electrodes have been soaked in water for about a month, after which any further change takes place very slowly.[3]

5. The liquid junction potential. The liquid junction between the medium in the flask *F* of figures 1 and 2 and the 3.5 N KCl of the reference calomel electrode is made, as already stated, by means of the dense porous plug *J*. The tube *C'–J* shown in figure 2 is sterilized with the flask, after which it is filled with 3.5 N KCl, and the connecting tube of the calomel electrode is inserted at *C'*. The porous plug *J* is made from a sintered mixture of 30 per cent alundum powder and 70 per cent finely ground Pyrex. With a head of about 15 cm. of the potassium chloride solution a

[3] In the more recent work we have used the "membrane" type of electrode described by MacInnes and Dole (MacInnes, D. A., and Dole, M., Ind. Eng. Chem., Anal. Ed., **1**, 57 (1929); Jour. Amer. Chem. Soc., **52**, 29 (1930)).

few drops a day will pass through the plug.[4] Experiment has shown that, within the limits of accuracy of the operation of the apparatus, such a liquid junction does not differ from one in which the 3.5 N KCl solution comes into direct contact with the medium. However, difficulty may be encountered in experiments, such as the calibration of glass electrodes, in which large changes of pH are involved, if the plugs are not cleaned before introduction into each solution.

This survey of the possible causes for irregularities in operation of the apparatus indicates that the only adjustment necessary is for a slow shift of the zero of the electrometer. The apparatus has, as a matter of fact, functioned for 100 hours with no other attention.

We have found that some simplification is possible in the flask *F*, leading to increased ease in sterilization. Portability of the apparatus might be attained by the use of a vacuum tube electrometer, and we are investigating that possibility. However, our tests up to the present, and a survey of the literature, indicate that such electrometers have not yet been made with the required stability in operation.

B. TESTS ON THE ACID PRODUCTION OF LACTOBACILLUS ACIDOPHILUS

As is well known the growth of *Lactobacillus acidophilus* is accompanied by the production of much acid, principally lactic, and consequently a rapid lowering of the pH of the medium. This organism was chosen for study with the aid of the apparatus described in the previous section. The stock culture, which was obtained from a pure commercial strain, was carried in litmus milk with frequent subcultures. The medium used in all of the experiments to be described was 2 per cent "bacto"-peptonized milk and 3 per cent galactose. The experiments were carried out at 38° in an oil thermostat.

Quite unexpected difficulty was encountered in our early

[4] These plugs are, by several tests, less porous than the finest Berkefeld filters, so that they cannot be sources of bacterial contamination.

attempts to grow the bacteria in flask *F* of the apparatus. Since pH regulation by the addition of alkali cannot be carried out without stirring, it seemed probable that the difficulty was in some way connected with the agitation of the medium. In the undisturbed medium the organism itself will adapt the conditions to favorable growth. This is shown by the familiar phenomena observed when the bacteria develop in a test-tube of litmus milk. The medium, initially blue, is first bleached to white, and the dissolved oxygen presumably largely removed. This stage is followed by the formation of a top layer of pink solution, where the medium is in contact with the air, due to diffusion of oxygen and to re-oxidation of the litmus. The pink color results, of course, from the lowering of the pH by the acid-forming bacteria. It is evident that if air is constantly stirred into the medium the initial reduction cannot occur, at least to the same extent. On the basis of these observations it was decided to try to grow the bacteria in the stirred medium in the absence of oxygen. These experiments were, however, completely unsuccessful. Very slight or no growth was observed when the air in the flask *F* was replaced by a stream of purified nitrogen or hydrogen. It was, however, observed that growth was always obtained if the regulations of pH were made with sodium carbonate; and more difficulty was experienced if the adjustments were made with sodium hydroxide. This observation is in accord with the conclusion of Theobald Smith (1924; 1926) and of Valley and Rettger (1927) that carbon dioxide is necessary for the growth of bacteria. In our tests with purified nitrogen and hydrogen the carbon dioxide was apparently swept out of the medium. These early experiments, which are very briefly summarized above, indicate that although a high tension of oxygen is not favorable to growth of *Lactobacillus acidophilus* it will not completely discourage it, whereas carbon dioxide is absolutely necessary.

Strangely enough, an attempt to grow these bacteria in the apparatus in an atmosphere of pure carbon dioxide at pH 7 resulted in a very slight growth. This result is also due to the fact that the stirred medium tends to come into equilibrium with the

atmosphere above it. The most important chemical equilibrium involved is

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

for which there is the mass action expression

$$\frac{(H^+)(HCO_3^-)}{(CO_2)} = K = 4.8 \times 10^{-7} \text{ (5) at } 38°$$

in which the parentheses represent activities of the substances enclosed. Thus, at a given hydrogen ion activity, or constant pH, there will be a constant ratio of the activities of the bicarbonate ion constituent and the dissolved carbon dioxide, and the latter is, of course, related to the partial pressure of gaseous carbon dioxide by Henry's law. Thus at pH = 7 and 1 atmosphere of carbon dioxide, the solubility under these conditions being 0.02433 mol per liter (Van Slyke, Sendroy, Hastings and Neill, 1928), the bicarbonate ion activity is 0.117. In the apparatus the titrating fluid was automatically added until a bicarbonate concentration of this activity was reached. The concentration obtained directly from the amount of alkali added was found to be 0.30, indicating a not improbable activity coefficient. The salt concentration was apparently too high for effective growth of the bacteria.

Excellent growth was, however, obtained when the gas passed through the medium was about 10 per cent carbon dioxide by volume, the remainder being nitrogen and about 0.2 per cent oxygen, the latter being due to a small proportion of that gas in the high pressure tank of nitrogen from which the mixture was made. Before inoculation the nutrient medium was brought to equilibrium with this gas mixture at the desired pH. This equilibration before inoculation was carried out for as long as sixteen hours without the appearance of turbidity or other evidence of bacterial growth due to contamination. Some typical measurements of the acid production by the bacteria with and without pH control are given in figure 3 where the amounts of alkali, in milli-equivalents per liter of nutrient medium necessary to titrate the acid are plotted as ordinates against the time in hours

as abscissae. For the regulated pH values the ordinates were computed from the readings of the buret *B*. In obtaining the data for the curve for "no pH control" the bacteria were grown, in the conventional manner, in flasks, 5-cc. samples being removed at intervals after gentle shaking and titrated, using phenolphthalein as indicator. Two such flasks were used and gave closely agreeing values. It will be observed that the amount of acid produced in a given time is greatly increased by keeping the pH value at 7, instead of leaving it uncontrolled. After 50 hours this increase is of the order of 100 per cent. Still greater acid

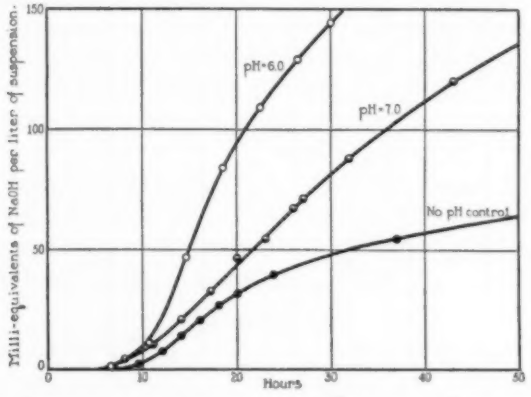

FIG. 3. ACID PRODUCTION OF LACTOBACILLUS ACIDOPHILUS WITH AND WITHOUT pH CONTROL

production is obtained by regulating the pH at a value of 6, the increase over no regulation being of the order of 200 per cent. The acid production curve for pH = 5.5 lies very close to that for pH = 6, the optimum being apparently near the latter value.

Figure 4 shows the change of pH with time when the organism was grown in flask *F* of the apparatus, but without pH control. The same medium was used as in the experiments with control, and the same rate of stirring. The pH, initially adjusted to a value of 7.25, dropped rapidly after inoculation and reached the apparent optimum value of 6 after 9 hours. This rapid drop continued to about pH 4, after which the change was slower.

The lowered acid production without pH control is obviously explained by this pH trend. It is of interest in this connection that Topley and Wilson (1929) say that *L. acidophilus* "grows best" at pH 7.6. It would appear to us that this is not a true optimum, but merely that this high initial pH gives the organism a large range of pH values to pass through before attaining the limiting value of about 4. On the other hand, Weiss and Rettger (1934) in a recent paper find the "average optimum" of this organism to lie between the pH values 5.8 and 6.6, in substantial agreement with our observations.

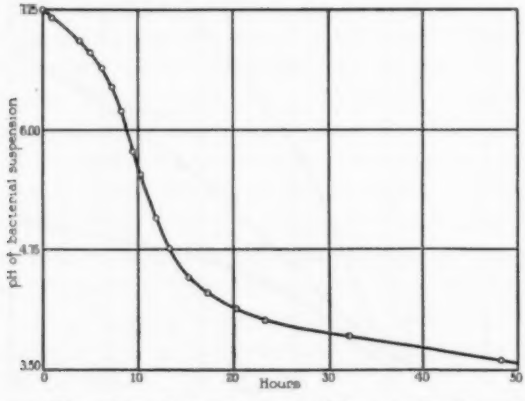

FIG. 4. CHANGE, WHEN NOT CONTROLLED, OF pH OF THE BACTERIAL SUSPENSION

In another series of experiments, which is still in progress, the measurements of acid production with no pH control and with control at various pH values have been accompanied by plate counts, photoelectric nephelometer readings, and determinations of apparent oxidation reduction potentials by means of an inert electrode. Such conclusions as are possible from our experiments as to the physiology of bacterial growth will be considered in a later paper.

SUMMARY

An apparatus is described by means of which the pH of a nutrient medium can be accurately and automatically controlled

during bacterial growth. The apparatus depends upon the measurement of the pH with a glass electrode, and the control of the addition of a solution of alkali by means of a photoelectric relay which is actuated by the deflection of a beam of light by the mirror of an electrometer. The apparatus functions for indefinite periods with a minimum of attention.

The apparatus has been used in the study of the acid production during the growth of *Lactobacillus acidophilus* at different controlled pH values and, for comparison, with no pH control. It has been found that the acid production can be greatly increased by maintaining the pH at relatively low values. It has, however, been found necessary to study the effect of the dissolved gases on the bacterial growth. Our studies have confirmed the conclusion of other workers that, at least for the organism under observation, carbon dioxide is necessary for such growth.

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APPLICATION OF STATISTICS TO PROBLEMS IN BACTERIOLOGY

IV. EXPERIMENTAL COMPARISON OF THE DILUTION METHOD, THE PLATE COUNT, AND THE DIRECT COUNT FOR THE DETERMINATION OF BACTERIAL POPULATIONS

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In previous publications (1933 a, b and c) we have presented certain theoretical considerations in regard to the dilution method of estimating bacterial populations. In the first of these publications, equations were developed for use in the evaluation of dilution data. The second publication was concerned with the accuracy of data obtained by using several tubes of a single dilution. The third paper was concerned with the accuracy of data obtained when several tubes of each of several dilutions were used. It was shown that with several dilutions it is possible to evaluate the limits of accuracy of the data obtained. To check the theoretical considerations with experimental data, we have determined bacterial populations by means of the dilution method, the plating method, and the direct count in order to compare the results obtained by the three methods. This paper is concerned primarily with the presentation of these experimental data.

METHOD

To determine the population of a heavy suspension of bacteria, it is necessary first to dilute the original suspension. The least dilution is required for the direct microscopic count, and the greatest for the dilution method. Sterile tap water adjusted to pH 7.0 was used as a diluent. We were guided in this by the work of Wilson (1922). To avoid the error which may occur as a result of evaporation of the measured diluent during autoclaving, the

sterile tap water was measured aseptically into sterile Erlenmeyer flasks by means of a 99-cc. sterile pipette or a 50-cc. sterile burette, depending upon the volume of water desired.

A uniform suspension of the material to be examined was secured for all experiments by shaking it for five minutes with $\frac{1}{8}$-inch glass beads in a 500-cc. Erlenmeyer flask. In addition, the first dilution of the material was shaken with beads for about two minutes. Stein (1918) found that shaking of crude sewage for longer than five minutes did not further increase the bacterial count.

Dilutions were made in powers of 10. For this purpose, 10-cc. volumetric pipettes graduated to deliver 0.1 cc. were used for measuring amounts greater than 1 cc., and 1-cc. volumetric pipettes were used when only 1 cc. was required. The first dilution was always made by adding 10 cc. of the material to 90 cc. of sterile tap water. Further dilutions were made by adding 1 cc. of this initial dilution to 99 cc. of diluent. Intermediate dilutions of 1:10 were made by adding 5 cc. of one dilution to 45 cc. of sterile water to secure the dilution with the next higher power of 10.

The direct count. For the direct count we used a combination of the method of Breed and Brew (1925) and that of Benians (1916), as described by Henrici (1923). One cubic centimeter of the 1:10 dilution of the bacterial suspension was thoroughly mixed with an equal volume of a 2 per cent aqueous solution of Congo red. Instead of using ruled squares on a slide, it was found more convenient to spread the mixture over a definite circular area. The circular area can be outlined on a glass slide more accurately than a square. For this purpose circles having a radius of 1.261 cm. were inscribed on 1.5-by-3-inch glass slides by means of a pair of dividers, to one arm of which a diamond pointed pencil was attached. The diameter was checked by means of a mechanical stage which could be read to 0.01 cm. These circles then had an area of 5 sq. cm. With a calibrated capillary pipette, 0.01 cc. of the bacteria Congo-red mixture was removed and spread as uniformly as possible over the circular area. The use of the circular area instead of a square removed the difficulty of spreading the material into corners. This should

increase the accuracy of the method. The slides were dried in the air, after which they were fixed in a 1 per cent solution of hydrochloric acid in 95 per cent alcohol, which also served to convert the dye to the blue form. When the alcohol had evaporated, the bacteria were counted.

Counting was facilitated by the use of an eyepiece micrometer which was ruled in 10 squares each way, thus making 100 small squares. This eyepiece micrometer was used with a Leitz oil immersion objective (1/12) and a no. 5 ocular. The eyepiece micrometer was calibrated against a stage micrometer. For this calibration, we used a tube of such length that a distance of 0.1 mm. on the stage micrometer corresponded to the width of the large square of the micrometer eyepiece. Thus the area of the eyepiece micrometer represented an area of 0.0001 sq. cm. on a slide to be examined. Since the area of the bacterial film to be examined was 5 sq. cm., the total number of fields in this area was 5/0.0001. The average number of bacteria (n) per field (large square) times the number of fields gives the number of bacteria in the volume (1/200 cc.) of the 1:10 suspension used in making the film. Then $(n \times 5 \times 200)/0.0001 = n \cdot 10^7$ bacteria per cubic centimeter in the dilution used in making the slides, or $n \cdot 10^8$ for the undiluted material.

Usually 100 fields were counted on each slide along the diameter of the circular area, although sometimes 50 fields were counted on each of two slides. The number of slides made and the number of fields counted varied with the different experiments.

The plating method. The Koch plating method was used. Petri plates were inoculated by delivering 1 cc. of the proper dilution into each plate from a 10 cc. volumetric pipette graduated in 0.1 cc. Standard beef extract agar was melted and brought to a temperature of 42°, ±1°C. The agar was then poured into the inoculated plate and rotated several times to secure a uniform distribution of the inoculum in the agar. After the agar had solidified, the plates, with one exception to be mentioned later, were inverted and incubated at 37°C. for forty-eight hours. All the colonies on the plate were counted by the aid of a Wolffhuegel counting apparatus.

The dilution method. Sets of ten tubes or multiples of ten tubes

were inoculated with each of three dilutions. The inoculation of large numbers of tubes was facilitated by using a test tube rack similar to that used by McCrady (1915) and by Wells (1918). These racks were made of heavy tin and were of such a size as to hold six 150-by-16 mm. straight-walled culture tubes. The inside dimensions of these racks were 10 cm. long, 1.6 cm. wide, and 10 cm. deep. The sides were open except for a strip of tin at the top, 4.5 cm. wide. The cover was 10.2 cm. long, 1.9 cm. wide, and 2.5 cm. deep. A layer of cotton was cut to fit snugly in the top of the cover. Similar racks containing 11 tubes have been used in later work.

Because of variation in the diameter of the tubes, the cover was made of the above size, which enabled us to remove and replace it easily. The cover was held in position by a heavy rubber band. The rack, as shown in figure 1, has several advantages over the types formerly described. The tubes are held in a single row, and the bottoms are clearly visible, permitting positive and rapid observations. This is particularly advantageous in the case of certain organisms which form a sediment.

The medium used depended upon the organism or type of material studied. In the present investigation, only liquid mediums were used. About 10 cc. of the medium were placed in each tube. The apparatus was sterilized at 15 pounds for fifteen minutes.

The dilutions were so selected that the volume of the inoculum remained constant at 1 cc. This tended to decrease errors caused by the measurement of small volumes. In fact, if smaller volumes of the same dilution are used instead of the same volume of the next higher dilution, considerable error may be introduced because of the greater chance that organisms may adhere to the wall of the culture tubes in the process of inoculation. The sixth tube in each rack was used as a control to determine the chance of contamination when using this type of rack. Of the 6100 tubes used in the course of this work, 1160 were controls. Two of these controls were found to be contaminated. By using the system described above, it was possible to inoculate about 5 tubes per minute. The inoculated tubes were incubated at 37°C. for forty-eight hours, except in a special case to be mentioned later.

For the first part of this study it was desirable to use an organism which was known to give a uniform suspension and as accurate a plate count as possible. Some preliminary tests were made to determine the dilution which would give a plate with an optimum number of colonies, and also to determine the dilutions which would, respectively, cause growth in nearly all tubes, cause growth in about half the tubes, and cause growth in but few of the tubes. To obviate the daily repetition of these preliminary

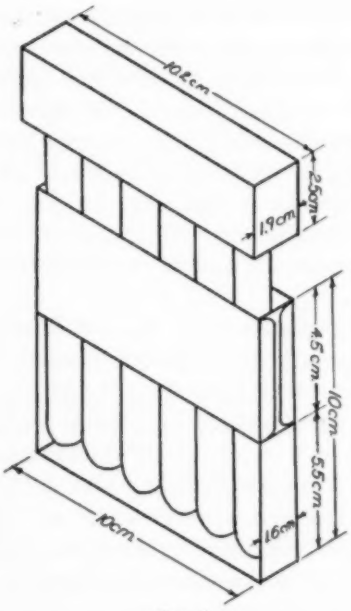

FIG. 1

tests, we used a culture which had been transferred daily to a 1 per cent peptone solution for a period of thirty days. In this way a nearly constant population of the organism was maintained in the twenty-four-hour cultures. For example, when *Escherichia coli* was the test organism, preliminary experiments showed that the desired results were obtained when 6 cc. of a 1:10 dilution of a twenty-four-hour culture were added to 94 cc. sterile tap water, and this diluted to 10^5 for plating, and to 10^6, 10^7, and 10^8 for the

dilution method. The dilutions used in the experiments are given in the appendices.

EXPERIMENT 1

From the 1:10 dilution of the twenty-four-hour *E. coli* culture, three 1-cc. samples were removed and each mixed with 1 cc. of Congo red for the direct count. From each of these mixtures, 10 slides were made as described above. A hundred fields were counted on each of these slides. The data are given in Appendix I.

Six cubic centimeters of the same 1:10 dilution of the twenty-four-hour *E. coli* culture were added to 94 cc. of sterile tap water. Further dilutions were made as described earlier. Ninety-two 1-cc. samples were removed from the 10^5 dilution for plating as described. One hundred sixty 1-cc. samples were removed from the 10^6 dilution and inoculated into 16 sets of 10 tubes each containing peptone solution. Similarly, 16 sets were inoculated from the 10^7 and 10^8 dilutions. The results of these experiments are found in Appendices II and III respectively for plate counts and dilution data.

Of the tubes inoculated from the 10^6 dilution, 158 showed growth and 2 no growth; from the 10^7 dilution, 61 tubes showed growth and 99 no growth; from the 10^8 dilution, 8 tubes showed growth and 152 no growth. The most probable number of organisms present in 1 cc. of the 10^7 dilution, as determined by means of equation 8 in earlier publications (1933 a, b), is found to be 0.4669. It has also been shown in an earlier publication (1933c) that the likely limits of accuracy can be evaluated. We find in this experiment that the actual population in the 10^7 dilution of the stock suspension, within reasonable probability, lies between 0.389×10^7 and 0.584×10^7. In the appendices this is referred to as the range. The values are determined from the curve presented in the third paper of this series. Since the stock suspension represents a $1:166\frac{2}{3}$ dilution of the original suspension, the population of the suspension, as determined by the dilution method, is 0.7782×10^9, and the limits of variation are 0.759×10^9 and 0.954×10^9.

From the direct counts given in Appendix I, we have calculated

the mean values of the individual slides. These are presented in table 1. The mean of the 30 slides is 17.054. The number of organisms per cubic centimeter in the original material is then 1.705×10^9. From the standard deviations of the means of 30 slides, the limits of variation based upon a criterion of twice the standard deviation have been calculated. Therefore, from the direct count data, we find that the actual population lies somewhere between 1.618×10^9 and 1.792×10^9.

TABLE 1

Mean values of individual direct counts of Escherichia coli in experiment 1

12.69	16.23	15.65
21.22	15.96	17.03
12.59	14.12	20.06
20.68	17.07	16.39
19.07	14.49	16.75
19.78	11.97	20.01
17.30	16.37	17.14
15.04	15.86	18.88
17.58	19.46	18.85
17.57	18.92	16.90

N	= 30
Mean	= 17.054
Standard deviation of means of individual slides	= ±2.3563
Coefficient of variation of means of individual slides	= 27.63 per cent
Standard deviation of means of 30 slides	= ±0.4376
Coefficient of variation of means of 30 slides	= 5.13 per cent

The values obtained from the plates are found in Appendix II. It is to be observed that the mean value of 92 plates is 48.72 colonies per plate with a 10^5 dilution of the stock suspension. The standard deviation of the mean is ±0.857. Again, using twice the standard deviation as a criterion of accuracy, we find that the actual population in the stock suspension as determined by the plate method is somewhere between 47.0×10^5 and 50.4×10^5.

The stock suspension represents a dilution of $1:166\frac{2}{3}$ of the original suspension. Therefore the number of organisms per cubic centimeter in the original suspension, as determined by the plate count is 0.812×10^9. The true value in the original sus-

pension will then lie somewhere between 0.789×10^9 and 0.840×10^9. The results are summarized in table 2.

It can be seen from this table that the values obtained by the dilution and plating methods agree, but that the direct count gives a value which is significantly higher.

EXPERIMENT 2

This experiment was essentially a repetition of experiment 1, except that a pure culture of *Bacillus subtilis* was used instead of *Escherichia coli*, and no direct count was made. *B. subtilis* was grown in beef extract broth, and transferred daily for 15 to 20 generations in order to obtain a uniform actively growing culture with a relatively constant population. The plates and tubes were inoculated as in the previous experiment. The medium used for the dilution experiment was beef-extract broth adjusted to pH 7.2. Because of the aerobic nature of the organisms to be used, only 5 to 6 cc. of medium were placed in each tube. The results of these experiments are found in Appendices II and III.

The data were analyzed in the same manner as for experiment 1. The results are summarized in table 3. These data show, as in experiment 1, that there is no significant difference in the values obtained by the two methods.

In the two following experiments, mixed cultures were used. It is well known that some bacteria are antagonistic to others. *Pseudomonas pyocyanea* is known to exert this antagonistic effect on *E. coli*. It is a common opinion that on plates made from mixed cultures containing nearly equal numbers of different organisms, one or more of which is antagonistic to the others, colonies of one predominate. It would appear that the plate counts of such mixtures would be abnormally low. It was therefore considered of importance to determine whether the dilution method would give a better measure of bacterial population than the plating method in such a mixed culture.

Experiment 3 was therefore done on a mixture of two organisms, one of which is known to be antagonistic to the other. As a control, experiment 4 was carried out with a mixture of two

organisms which are supposed to be non-antagonistic toward each other. In these experiments determinations were made not only on the mixed cultures but also on the pure cultures of the individual organisms before they were mixed in order to secure accurate information regarding the number of each kind of organism present in the mixture. For experiment 3, twenty-four-hour cultures of *E. coli* and *Pseudomonas pyocyanea* in peptone solution were used. For experiment 4, twenty-four-hour cultures

TABLE 2

Summary of experiment 1

METHOD	MOST PROBABLE POPULATION PER CUBIC CENTIMETER IN ORIGINAL CULTURE	REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
Direct count	1,705,000,000	1,618,000,000–1,792,000,000
Plate count	812,000,000	783,000,000– 840,000,000
Dilution method	778,200,000	759,000,000– 954,000,000

TABLE 3

Summary of experiment 2

METHOD	MOST PROBABLE POPULATION PER CUBIC CENTIMETER IN ORIGINAL CULTURE	REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
Plate count	142,000,000	137,000,000–146,000,000
Dilution method	172,000,000	135,000,000–214,000,000

of *E. coli* and *Staphylococcus aureus* were used. As in previous experiments, each culture was transferred daily into peptone solution, except that beef extract broth was used for *S. aureus*.

EXPERIMENT 3

Twenty-four-hour cultures of *Ps. pyocyanea* and *E. coli* in peptone solution were each shaken with beads for five minutes. Ten cubic centimeters of the *E. coli* culture were then added to 90 cc. of sterile tap water in a 500-cc. flask containing beads. Additional dilutions were made as indicated by preliminary experiments. The remainder of the 1:10 dilution was saved for the mixed culture. Five cubic centimeters of the culture of *Ps.*

pyocyanea were added to 45 cc. of sterile tap water and shaken as usual, after which, further dilutions were made.

The mixed culture was prepared by adding 5 cc. of the 1:10 dilution of *E. coli*, prepared above, and 12 cc. of the original culture of *Ps. pyocyanea* to 83 cc. of sterile tap water. This mixture was shaken for five minutes. One cubic centimeter was then added to 99 cc. of water, and again shaken for five minutes.

The part of this experiment involving the pure culture of *E. coli* will be referred to as experiment 3a, and that involving *Ps. pyocyanea* as experiment 3b. The data from these experiments are given in Appendices II and III. The data were analyzed, as for the previous experiment,and the results are summarized in table 4.

The values summarized in table 4 under the heading "mixed culture calculated from pure culture data" were obtained by calculating the number of organisms that should have been present in the mixture from data obtained in the pure cultures. For example, in this mixture 5 cc. *E. coli* and 12 cc. *Ps. pyocyanea* were added to sterile tap water to make a total volume of 100 cc. From the plate counts, then, the mixture should have contained

$$\frac{0.5 \times 1.343 \times 10^7 + 12 \times 0.2023 \times 10^9}{100}$$ organisms per cubic centimeter

This gives 30,990,000 organisms per cubic centimeter in the mixture.

For the mixed culture, the dilution data give a value significantly greater than that obtained by the plating method. The same results are obtained in the pure culture of *Ps. pyocyanea*. With the pure culture of *E. coli*, however, the results obtained by the two methods are in agreement, as was found in experiment 1. There has been an apparent decrease in viable organisms in the mixed culture as compared with the values obtained for the pure cultures. These changes are sufficiently great to be significant, regardless of the method of counting. This decrease in the number of viable organisms is not explainable on the basis of longer contact of organisms in the mixed cultures because the inoculations were made first from the mixed culture. It is possible that some growth may have taken place in the pure culture during the time required to complete the inoculation of

the mixed cultures. We believe that this would not account for the entire discrepancy since the pure cultures were kept in the ice box during the three hours required to inoculate the mixed culture. Furthermore, the cultures were cooled to ice box temperature before the mixture was made. It is not possible, from these data, to determine whether this decrease in viable organisms is due to the toxic effect of *Ps. pyocyanea* upon *E. coli* or upon itself.

TABLE 4

Summary of experiments 3, 3a and 3b

METHOD	MOST PROBABLE NUMBER OF ORGANISMS PER CUBIC CENTIMETER		REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
	Found experimentally	Calculated from pure culture data	
Mixed culture			
Plate count	16,730,000	30,990,000	16,140,000– 17,320,000
Dilution method	36,420,000	61,390,000	30,350,000– 45,500,000
Pure culture of E. coli			
Plate count	1,343,000,000		1,200,000,000–1,390,000,000
Dilution method	1,118,000,000		760,000,000–1,803,000,000
Pure culture of Ps. pyocyanea			
Plate count	202,300,000		175,300,000– 229,300,000
Dilution method	465,000,000		314,100,000–1,453,000,000

A discrepancy is observed between the plate count and the dilution count in both the pure culture of *Ps. pyocyanea* and in the mixed culture. Since the mixture contains many more *Ps. pyocyanea* than *E. coli*, the discrepancy for the mixture may be caused by the same factors which are operating in the pure culture of *Ps. pyocyanea*.

EXPERIMENT 4

This experiment was done as a control for experiment 3. *E. coli* and *S. aureus* were used because they are not ordinarily considered to be antagonistic toward each other when grown together

in a mixed culture. *S. aureus* was subcultured on beef-extract broth and *E. coli* on peptone solution. Beef-extract broth was used in this experiment for the dilution method in order to provide a medium in which both organisms would grow well. Standard nutrient agar was used for plating. The proper dilutions for the individual pure cultures and for the mixed cultures were determined by preliminary experiments. The mixed culture was made by adding 5 cc. of the *E. coli* culture and 7 cc. of the *S. aureus* culture to 88 cc. of sterile tap water. The parts of this experi-

TABLE 5

Summary of experiments 4, 4a and 4b

METHOD	MOST PROBABLE NUMBER OF ORGANISMS PER CUBIC CENTIMETER		REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
	Found experimentally	Calculated from pure culture data	
Mixed culture of *E. coli* and *S. aureus*			
Plate count	54,750,000	61,170,000	53,000,000– 56,500,000
Dilution method	54,880,000	68,368,000	45,700,000– 68,600,000
Pure culture of *E. coli*			
Plate count	767,000,000		713,000,000– 821,000,000
Dilution method	1,030,800,000		701,000,000–1,663,000,000
Pure culture of *S. aureus*			
Plate count	326,400,000		306,000,000– 347,000,000
Dilution method	240,400,000		164,000,000– 388,000,000

ment dealing with pure cultures of *E. coli* and *S. aureus* will be designated as experiments 4a and 4b respectively. The results are given in Appendices II and III. The data of experiment 4 are summarized in table 5.

The data obtained in this experiment are in agreement both on the pure cultures and on the mixed cultures. There is, however, as in experiment 3, some indication of a decrease in the number of viable organisms in the mixed culture. The decrease in this case, however, may not be sufficiently great to be significant.

EXPERIMENT 5

In experiment 3, with a mixed culture of *E. coli* and *Ps. pyocyanea*, we found that the dilution method gave higher values than the plate count. This was also true with the pure culture of *Ps. pyocyanea*. The mixture contained about five times as many *Ps. pyocyanea* as *E. coli*, so the lack of agreement in the case of the mixture might have been due to the excessive numbers of this organism. To verify this point, we repeated experiment 3, but with a mixture containing about equal numbers of the two species.

For experiment 5, suspensions of *E. coli* and of *Ps. pyocyanea* were prepared as in experiment 3. The mixed culture was prepared by adding 5 cc. of each of the above cultures to 90 cc. of sterile tap water. The mixture was shaken for five minutes. Additional dilutions of 10^5 for plating and 10^6, 10^7, and 10^8 for the dilution method were made.

The part of this experiment involving the pure culture of *E. coli* will be referred to as experiment 5a, and that involving *Ps. pyocyanea* as experiment 5b. The data resulting from these experiments are found in Appendices II and III, and are summarized in table 6.

The tendencies revealed in experiment 3 are apparent in the above data. With the pure culture of *Ps. pyocyanea* the dilution method gave counts significantly higher than the plate count. With the pure culture of *E. coli*, there is again a fair agreement in the results of the two methods. This time, however, the mixture contained a few more *E. coli* than *Ps. pyocyanea*, and the difference between the dilution method and the plate method is not sufficiently great to allow us to state that it is significant. It appears from this that the discrepancy between the two methods, as observed in experiment 3, was due to the fact that the mixture contained considerably more *Ps. pyocyanea* than *E. coli*. The discrepancy is apparently not due to the toxic effect of *Ps. pyocyanea* on *E. coli* but is rather a phenomenon which is characteristic of *Ps. pyocyanea* itself.

We were interested in determining whether the discrepancy between the calculated number of organisms in the mixture and

the number found in experiment 3 could be due to *Ps. pyocyanea* alone. In doing experiment 5, therefore, a record was kept of the number of tubes which showed a green pigment indicating growth of *Ps. pyocyanea*. All other tubes were regarded as showing no growth in respect to this organism. The results are recorded in table 7.

In table 8 the population of *Ps. pyocyanea* in the mixture from experiment 5 was obtained as a direct value by counting the number of tubes which showed a green pigment. This is recorded

TABLE 6

Summary of experiments 5, 5a and 5b

METHOD	MOST PROBABLE NUMBER OF ORGANISMS PER CUBIC CENTIMETER		REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
	Found experimentally	Calculated from pure culture data	
Mixed culture			
Plate count	21,500,000	28,570,000	20,800,000– 22,200,000
Dilution method	23,410,000	30,900,000	19,500,000– 29,500,000
Pure culture of *E. coli*			
Plate count	435,400,000		388,000,000–483,000,000
Dilution method	379,000,000		258,000,000–611,000,000
Pure culture of *Ps. pyocyanea*			
Plate count	136,000,000		126,000,000–146,000,000
Dilution method	239,000,000		163,000,000–386,000,000

as *Ps. pyocyanea* experiment 5c. In experiment 5 we obtained the total number of organisms. The difference between the total number of organisms and the number of *Ps. pyocyanea* we assume to be *E. coli*, and it is recorded in table 8 as *E. coli*, experiment 5c. The *Ps. pyocyanea* population was also calculated from data obtained with the pure culture. The number that should have been present in the mixed culture, as calculated from these data, is recorded in table 8 as *Ps. pyocyanea*, experiment 5b. Likewise, the population of *E. coli* which should have been present in the mixed culture is recorded as the coli population in experiment 5a.

It is apparent that the *Ps. pyocyanea* populations as calculated and as found experimentally are in agreement, while the corresponding *E. coli* populations do not agree. The data show that when *E. coli* and *Ps. pyocyanea* are mixed, there is an immediate

TABLE 7

Dilution data showing growth and no growth of Ps. pyocyanea in mixed culture used in experiment 5

SET	10^8 p_1 q_1	10^7 p_2 q_2	10^6 p_3 q_3
1	10–0	6–4	2–8
2	10–0	6–4	0–10
3	10–0	5–5	1–9
4	10–0	9–1	0–10
5	9–1	7–3	1–9
6	10–0	8–2	0–10
7	10–0	6–4	0–10
8	10–0	4–6	0–10
9	9–1	6–4	2–8
10	10–0	6–4	1–9
11	8–2	6–4	0–10
12	10–0	5–5	2–8
13	10–0	5–5	1–9
14	10–0	7–3	0–10
15	10–0	7–3	0–10
16	10–0	6–4	1–9
Totals.....	156–4	100–60	13–147

Calculated mode = 1.57
Range = 1.31–1.98

TABLE 8

Table showing the relationship between the experimental and calculated values for the bacterial populations studied in experiments 5, 5a and 5b

	MOST PROBABLE NUMBER OF ORGANISMS PER CUBIC CENTIMETER IN MIXTURE	REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
Experiment 5a: *E. coli* (calculated value)..	18,900,000	12,900,000–30,500,000
Experiment 5c: *E. coli* (by difference).....	7,710,000	6,400,000– 9,700,000
Experiment 5b: *Ps. pyocyanea* (calculated value).....	11,960,000	8,140,000–19,300,000
Experiment 5c: *Ps. pyocyanea* (experimental value).....	15,700,000	13,100,000–19,800,000

destruction of a large number of *E. coli*, and that this destruction is sufficient to account for the entire discrepancy between the number of organisms found in the mixture and the number which should have been present, as apparent from data calculated from the respective pure cultures.

EXPERIMENTS 6 TO 16

In order to obtain additional data on the accuracy of the dilution method as compared with the plate count and the direct count, a series of experiments was carried out on suspensions of *E. coli* containing different numbers of bacteria per cubic centimeter.

Experiments 6 to 9 inclusive were carried out in the following manner. A twenty-four-hour culture in peptone solution was shaken as in previous experiments to secure a uniform suspension. A 1:10 suspension was then made by adding 10 cc. of the culture to 90 cc. of sterile tap water. From this suspension, 1, 2, 3, 4, and 5 cc. were removed and each diluted to 100 cc. in sterile tap water. Each of the five dilutions was then used as a stock suspension for a series of dilutions, of which the suitable ones were used for making plates and for the inoculation of tubes in the dilution method. Four plates and four sets of 10 tubes in 3 dilutions were used in each determination. From the 1:10 dilution of the original culture, one 1-cc. sample was removed, and slides 31, 32, 33 and 34 were made for the direct count, as described previously. On each of these slides, 100 fields were counted.

Experiments 10 to 16 were carried out in the same manner, except that another twenty-four-hour culture was used, and amounts of the 1:10 dilution varying from 6 cc. to 11 cc. were diluted to 100 cc. as a starting point for the additional dilutions needed. Determinations of the bacterial population were made on each of these, as in the former experiments. From the original 1:10 dilution of this series, two 1-cc. samples were removed, and two slides made from each for the direct count. These were numbered 35a, 35b, 36a, and 36b. Fifty fields were counted from each slide. The data obtained from these experiments are given in Appendices I, II, and III, and are summarized in table 9.

TABLE 9

Summary of experiments 6 to 16

EXPERIMENT NUMBER		MOST PROBABLE NUMBER OF ORGANISMS PER CUBIC CENTIMETER OF STOCK SUSPENSION	REASONABLY CERTAIN LIMITS OF THE TRUE VALUE	NUMBER OF ORGANISMS PER CUBIC CENTIMETER OF ORIGINAL CULTURE, AS CALCULATED FROM EACH EXPERIMENT
6	Direct count	1,611,000	1,339,000– 1,883,000	1,611,000,000
	Plate count	1,010,000	915,600– 1,104,400	1,010,000,000
	Dilution method	846,900	576,100– 1,366,000	846,900,000
7	Direct count	3,222,000	2,678,000– 3,766,000	1,611,000,000
	Plate count	2,307,500	2,213,100– 2,401,900	1,153,750,000
	Dilution method	2,528,000	1,720,000– 4,077,000	1,264,000,000
8	Direct count	4,833,000	4,017,000– 5,640,000	1,611,000,000
	Plate count	3,925,000	2,981,000– 4,869,000	1,308,300,000
	Dilution method	3,991,000	2,715,000– 6,437,000	1,330,300,000
9	Direct count	6,444,000	5,356,000– 7,532,000	1,611,000,000
	Plate count	7,700,000	6,756,000– 8,644,000	1,925,000,000
	Dilution method	8,833,000	6,009,000–14,247,000	2,208,250,000
10	Direct count	8,055,000	6,695,000– 9,415,000	1,611,000,000
	Plate count	8,375,000	7,431,000– 9,319,000	1,675,000,000
	Dilution method	7,850,000	5,340,000–12,661,000	1,570,000,000
11	Direct count	8,826,000	6,000,000–11,652,000	1,471,000,000
	Plate count	9,150,000	8,206,000–10,094,000	1,525,000,000
	Dilution method	7,810,000	5,310,000–12,600,000	1,301,700,000
12	Direct count	10,297,000	7,060,000–13,594,000	1,471,000,000
	Plate count	10,925,000	9,981,000–11,869,000	1,560,700,000
	Dilution method	9,490,000	6,460,000–15,310,000	1,355,700,000
13	Direct count	11,768,000	8,000,000–15,536,000	1,471,000,000
	Plate count	12,125,000	11,181,000–13,069,000	1,515,600,000
	Dilution method	16,740,000	11,390,000–27,000,000	2,093,300,000
14	Direct count	13,239,000	9,000,000–17,478,000	1,471,000,000
	Plate count	14,675,000	13,731,000–15,619,000	1,630,600,000
	Dilution method	13,880,000	9,440,000–22,239,000	1,542,200,000
15	Direct count	14,710,000	10,000,000–19,420,000	1,471,000,000
	Plate count	17,475,000	16,531,000–18,419,000	1,747,500,000
	Dilution method	13,850,000	9,420,000–22,340,000	1,385,000,000
16	Direct count	16,181,000	11,000,000–21,362,000	1,618,100,000
	Plate count	18,925,000	17,981,000–19,869,000	1,865,400,000
	Dilution method	18,610,000	12,660,000–30,020,000	1,691,800,000

In the first two experiments of this series, 6 and 7, the direct count gives a value significantly greater than that obtained by the other two methods. This is in agreement with the results obtained in experiment 1. In experiments 8 to 16, the results of the three methods agree. There is a possibility here that slight growth may have favored the count obtained by the plate and dilution methods. However, we believe that the agreement in these latter experiments is due to a more efficient breaking up of clumps because of the fact that larger amounts of the original stock suspension were used. This appears to be the case because experiments 11 and 12 are comparable to 6 and 7 in that no appreciable time was permitted during which growth could occur. Nevertheless, in experiments 11 and 12, there is an agreement between the direct count and that obtained by the other methods. These data suggest that the most accurate results are to be expected when a comparatively large amount of the original culture is used in making the stock or starting suspension. This dilution should then be shaken with beads before further dilutions are made. When this precaution is taken, we believe that the direct count will give results in agreement with those of the plate count and dilution method, provided the comparisons are made upon cultures before they have entered the death phase.

EXPERIMENT 17

As pointed out in the first publication of this series, our interest in this problem arose from a discrepancy observed between the plate count and the direct microscopic count on "curing pickle" used in packing houses. An experiment was therefore planned in which the plating method could be compared accurately with the dilution method to determine whether the latter would give the higher values.

The material chosen to be examined was curing pickle which had been in use about 30 days. The high salt content of this material, made it advisable to use a medium of similar composition. Used curing pickle which contained rather large amounts of salt and protein was obtained and sterilized in the autoclave. This precipitated the protein and at the same time clarified the

medium. The medium was then filtered, and the salt concentration was adjusted to 6 per cent by dilution with tap water. The sugar content was increased by the addition of about 1 gram of glucose per liter. The reaction was then adjusted to neutrality and part of the "salt broth" tubed for use in the dilution method. To another portion, 15 grams of Difco agar per liter were added to make a solid medium. After tubing, both the "salt broth" and "salt agar" were sterilized for twenty minutes at 15 pounds.

The material to be studied was diluted for use in the dilution method and for plating as indicated in Appendices II and III. Two series of plates were made, one using nutrient agar with a beef-extract base, and the other the "salt agar" described above. Because of the low temperature at which organisms associated with curing pickle are growing (3.3° to 4.4°C.), and their rather

TABLE 10

Summary of experiment 17

METHOD	MOST PROBABLE NUMBER OF ORGANISMS PER CUBIC CENTIMETER	REASONABLY CERTAIN LIMITS OF THE TRUE VALUE
Plate count (standard nutrient agar)	350,000	330,000– 372,000
Plate count (salt agar)	399,000	370,000– 428,000
Dilution method	680,000	462,600–1,096,800

slow growth, the plates and tubes in this experiment were incubated at 25°C. for four days. The plates could not be left longer than this because of overgrowth of molds.

The data obtained in this experiment are given in Appendices II and III, and are summarized in table 10.

The data show that salt agar gives a higher count than standard nutrient agar and that the dilution method gives a count which is significantly higher than the plate count on salt agar.

CONCLUSIONS

The experimental data presented show that the plate count and dilution counts give the same results on the pure culture studied, with the exception of *Ps. pyocyanea*. In the case of *Ps. pyocyanea* and of certain mixed cultures of *Ps. pyocyanea* and

E. coli, and in the case of organisms found in curing pickle, the dilution method gives higher values than the plate count.

If proper care is taken to break up clumps of bacteria, the direct count gives the same value as the plating and dilution methods when used upon cultures before they have entered the death phase. The dilution method offers a new method of studying the antagonistic effect of one organism upon another. The fact that the dilution method agrees with other methods appears to us to justify the use of Poisson's series for the development of the fundamental equations found in earlier papers of this series.

SUMMARY

Experimental data are reported which have been obtained by direct microscopic count of bacterial populations, by the plate count, and by means of the dilution method. These determinations have been made on a pure culture of *E. coli*, *B. subtilis*, *Ps. pyocyanea*, and *S. aureus*, and upon mixed cultures of *E. coli* and *Ps. pyocyanea*, *E. coli* and *S. aureus*, as well as upon the mixed culture found in packing house curing pickle.

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APPENDIX I

Table of data obtained by direct count

SLIDE 1	SLIDE 2	SLIDE 3	SLIDE 4	SLIDE 5	SLIDE 6
11*10 16 13 18	18 14 14 12 11	2 13 10 10 14	6 13 6 12 10	8 6 8 7 6	10 8 10 11 8
9 10 17 11 21	10 8 7 10 15	7 7 5 10 19	15 11 12 14 7	9 7 7 11 10	11 12 19 13 13
14 14 9 13 15	13 14 6 9 5	23 19 19 14 17	18 16 13 19 16	23 16 19 19 12	13 11 16 21 22
20 15 17 23 10	8 5 9 10 9	22 15 24 18 12	2 15 18 10 6	25 20 14 22 20	23 33 30 21 21
10 17 14 23 17	10 15 10 12 20	20 24 21 17 24	14 16 10 12 24	12 26 33 33 24	24 23 24 26 34
23 13 10 14 10	10 8 8 22 24	21 16 21 22 19	20 21 14 22 13	38 23 26 27 20	39 35 25 27 35
7 18 11 18 24	23 33 29 17 32	13 9 13 18 27	12 10 18 24 11	17 33 17 32 36	34 35 41 29 25
26 19 18 21 19	45 38 31 26 28	11 8 18 10 15	25 24 24 17 16	23 27 18 21 17	31 23 29 23 26
16 8 13 16 12	20 22 39 27 15	17 8 14 9 10	17 33 29 17 13	19 16 19 12 13	17 23 14 5 10
10 18 19 18 16	24 18 18 27 42	20 12 15 13 16	23 31 21 27 35	12 14 8 15 16	11 17 11 5 8
8 15 19 11 13	21 34 16 17 22	29 16 15 17 19	24 29 32 35 23	22 18 12 8 13	13 10 6 7 7
10 7 14 8 10	21 20 16 9 9	25 18 25 19 22	16 22 16 18 20	15 15 11 14 11	15 6 11 17 21
13 6 11 12 6	16 13 8 13 11	15 23 18 40 47	23 26 26 32 34	10 19 9 10 15	19 21 24 18 15
9 4 5 11 6	16 22 26 10 12	21 30 40 23 26	28 25 28 24 27	12 16 15 11 21	18 16 14 14 19
12 7 8 5 7	10 11 9 16 10	24 13 11 10 7	17 31 20 14 19	8 20 17 14 16	13 17 21 24 15
7 5 10 7 11	9 7 12 13 10	9 9 9 7 7	45 41 40 40 34	25 20 19 15 16	22 13 11 13 14
13 9 15 13 17	9 14 14 10 12	7 13 7 6 11	25 38 30 30 20	18 14 16 14 13	8 18 22 10 10
18 17 12 15 4	15 7 19 13 16	5 6 10 6 11	21 28 20 37 32	11 12 5 10 10	13 4 9 17 14
9 9 7 17 8	16 18 16 21 27	16 12 12 13 10	25 20 29 23 21	10 7 11 18 13	9 12 7 6 2
5 7 10 9 14	16 9 12 8 12	14 7 20 9 15	31 12 14 17 18	8 6 8 16 13	4 10 10 4 25
N 100	100	100	100	100	100
A 12.69	16.23	15.65	21.22	15.96	17.03
B ±4.971	±8.383	±7.701	±8.705	±7.030	±8.690
C 78.35%	103.30%	98.42%	82.04%	88.09%	102.05%

SLIDE 7	SLIDE 8	SLIDE 9	SLIDE 10	SLIDE 11	SLIDE 12
7 5 5 4 4	15 15 10 10 11	43 26 29 39 19	19 11 10 16 17	17 17 21 24 15	9 6 7 6 12
6 10 13 15 6	10 10 16 12 18	30 25 36 16 22	9 15 13 18 22	17 19 19 19 8	9 14 9 6 12
12 11 9 21 13	25 13 9 5 8	15 16 18 22 24	11 13 16 22 12	11 15 13 12 11	19 15 12 11 22
13 17 10 12 7	5 9 6 12 7	18 26 20 20 20	6 14 11 8 18	7 9 7 17 20	13 18 20 14 16
7 14 17 9 8	5 9 7 13 9	18 21 17 13 22	14 15 15 11 23	17 14 28 19 27	21 16 23 20 32
11 3 16 14 17	16 6 19 11 8	36 38 21 28 23	18 22 16 11 10	12 17 15 11 19	16 15 23 23 20
18 23 11 28 16	13 14 14 14 13	16 19 34 18 19	19 20 17 15 24	8 14 30 17 30	19 20 23 21 19
10 10 13 14 17	11 16 13 14 18	30 38 17 14 20	14 26 15 15 13	23 17 17 19 22	29 19 18 28 16
16 17 18 11 9	24 24 20 23 17	14 18 25 23 26	8 11 9 11 18	29 26 42 29 27	16 13 17 19 17
8 13 15 10 10	25 25 22 23 41	35 42 50 35 32	14 19 8 15 10	12 18 33 19 17	22 22 24 31 14
11 9 4 4 2	28 23 31 23 20	20 5 16 8 12	21 24 16 24 18	27 19 13 20 20	13 23 33 20 15
11 8 12 9 8	15 23 17 28 35	14 22 20 19 21	20 23 23 31 23	16 18 16 13 12	15 14 18 16 20
11 8 11 22 15	24 16 15 10 16	20 23 17 33 33	20 24 26 16 10	20 18 16 10 13	9 14 15 25 8
17 20 10 13 10	10 7 15 17 8	19 19 21 24 31	28 21 28 32 29	16 17 22 10 20	17 13 19 18 13
12 15 10 17 19	13 11 14 15 12	24 41 25 34 32	26 36 31 31 28	36 16 17 31 18	17 21 11 25 16
12 16 17 14 14	20 5 10 6 5	4 7 10 5 4	38 28 33 32 30	14 12 13 15 14	26 22 20 18 9
13 21 14 10 12	17 8 10 3 14	9 4 9 8 6	33 30 36 28 35	11 5 12 12 9	13 16 20 16 16
15 9 20 11 13	11 5 11 11 11	9 6 8 12 13	29 29 19 20 28	6 9 8 8 4	15 19 22 12 16
12 15 14 13 17	14 9 9 13 8	10 9 12 8 11	28 39 21 41 27	7 11 11 7 14	18 15 22 11 20
9 15 13 24 25	12 12 12 8 9	9 9 8 9 8	34 29 25 19 11	5 13 14 16 9	15 12 12 17 13
N 100	100	100	100	100	100
A 12.59	14.12	20.06	20.68	16.39	17.07
B ±4.930	±6.911	±10.197	±8.272	±7.060	±5.501
C 78.31%	97.89%	101.66%	80.00%	86.11%	64.46%

SLIDE 13	SLIDE 14	SLIDE 15	SLIDE 16	SLIDE 17	SLIDE 18
8 6 6 6 7	14 19 9 24 15	5 5 13 7 6	8 6 9 11 8	18 20 5 13 21	14 14 15 11 13
10 5 9 9 7	10 12 19 17 26	6 5 8 10 9	10 12 14 6 10	17 12 13 9 12	13 8 8 20 33
11 6 4 19 11	18 13 13 14 24	12 7 12 14 15	9 9 9 13 15	8 11 13 24 14	21 25 32 28 26
4 3 1 8 6	33 17 26 17 19	7 8 13 14 11	13 14 16 14 14	10 17 6 10 16	19 18 19 19 16
12 13 15 17 10	23 22 21 23 22	12 13 13 26 18	6 13 8 7 13	17 3 8 5 12	17 7 19 18 12
11 15 19 15 18	16 22 16 25 17	13 25 19 25 38	8 10 9 14 10	18 18 23 10 18	21 17 14 17 28
16 16 27 14 11	16 22 25 24 19	49 17 21 28 29	12 6 8 7 8	21 16 24 21 14	15 19 23 32 16
8 30 26 23 22	32 21 15 14 15	29 34 38 46 41	11 5 5 10 17	18 15 10 10 5	14 11 25 29 28
35 28 32 20 23	17 17 19 13 19	35 34 33 40 36	10 15 15 17 24	10 9 7 16 13	24 22 22 34 30
30 20 15 19 23	23 13 22 19 24	35 38 33 31 39	16 13 14 18 13	17 13 25 13 27	33 21 11 11 16
13 26 25 19 21	22 11 25 12 16	44 38 27 39 28	18 16 14 10 13	44 46 40 30 33	22 26 28 23 21
19 19 16 20 4	21 23 18 23 22	34 21 28 29 32	10 18 6 12 16	18 20 28 26 24	21 14 17 17 9
15 20 9 18 21	28 13 15 19 27	32 36 20 25 22	31 26 21 23 32	33 23 24 17 19	21 8 23 12 15
10 10 8 5 32	25 21 22 22 23	16 19 16 23 23	11 20 22 20 15	21 15 29 25 25	11 13 11 11 9
18 25 3 16 9	17 24 28 12 18	19 17 19 11 19	7 21 10 10 3	28 20 34 18 15	10 11 7 12 13
11 12 13 20 7	28 24 11 16 24	16 18 10 8 12	9 7 4 16 4	3 4 7 10 9	11 19 11 18 20
11 7 11 18 24	23 21 17 21 23	13 17 11 9 12	5 0 2 5 3	13 8 4 8 3	15 22 29 17 27
13 12 13 12 15	16 19 12 15 23	9 10 11 11 6	5 9 13 22 17	12 6 7 12 13	22 29 30 27 33
20 5 14 13 9	16 19 22 10 12	5 12 11 5 13	8 10 10 4 7	15 8 13 23 24	30 42 20 23 39
14 16 8 11 10	18 17 12 7 19	9 7 10 9 10	18 13 7 19 13	32 24 34 33 45	27 35 31 26 27
N 100	100	100	100	100	100
A 14.49	19.07	19.78	11.97	17.30	20.01
B ±7.342	±5.111	±11.487	±5.944	±9.518	±7.934
C 101.34%	53.61%	116.15%	99.31%	110.03%	79.30%

N = number of microscopic fields counted.
A = mean number of bacteria per microscopic field.
B = standard deviation of the counts of individual fields.
C = coefficient of variation of the counts of individual fields.
* = number of organisms per field.

Table of data obtained by direct count—concluded

SLIDE 19	SLIDE 20	SLIDE 21	SLIDE 22	SLIDE 23	SLIDE 24
8* 9 15 16 5	9 22 9 9 10	6 7 8 5 7	20 14 12 14 17	5 7 6 8 11	8 12 13 12 6
9 8 11 10 21	13 9 10 12 12	5 5 3 11 7	16 13 13 15 9	5 10 13 7 7	13 12 17 13 12
21 19 7 12 13	18 12 17 26 25	11 11 6 12 5	4 6 16 18 11	8 7 9 10 5	10 16 16 16 29
7 9 12 11 17	26 21 31 28 32	5 6 9 16 11	15 21 15 17 15	12 12 14 18 14	13 14 20 15 12
14 15 14 11 15	22 19 17 22 17	10 15 13 13 10	10 9 17 11 19	17 15 15 17 14	21 18 25 11 32
22 29 25 16 19	21 12 15 21 12	10 14 17 19 13	26 37 29 29 31	16 17 23 22 25	21 24 24 11 27
18 20 17 18 22	20 23 17 18 27	19 15 13 16 19	21 15 14 15 17	22 15 27 16 14	20 30 28 25 18
20 15 18 12 19	18 26 22 8 9	16 12 14 18 14	18 13 15 18 18	26 18 23 20 22	29 22 21 20 34
12 13 11 10 13	9 9 13 8 11	14 26 17 13 23	9 11 13 15 9	26 20 31 15 20	25 22 22 17 20
14 19 10 21 16	15 12 14 7 11	14 12 19 24 19	10 13 7 8 8	17 26 22 19 23	18 14 9 15 16
19 20 7 14 13	12 13 21 16 14	22 21 23 21 19	13 16 12 9 17	18 27 35 28 27	19 20 10 13 13
19 15 10 6 13	23 22 24 25 15	19 20 28 25 19	13 15 16 22 25	33 21 24 23 24	13 18 22 18 21
19 8 19 17 11	31 19 29 28 30	24 24 24 31 30	24 17 27 17 15	21 25 24 29 22	20 17 17 15 4
20 13 24 24 22	23 20 24 21 17	25 27 35 20 25	20 26 22 16 22	27 32 18 28 34	20 15 13 20 8
37 26 34 29 25	36 34 17 12 12	19 17 14 26 16	15 26 24 24 23	23 23 36 20 31	20 17 12 14 9
17 15 20 13 14	10 10 11 13 10	14 20 9 6 8	21 12 21 10 12	23 22 15 23 19	11 18 16 18 17
15 23 12 15 16	14 8 8 14 22	6 8 14 9 9	9 13 10 12 9	32 20 13 24 22	13 10 21 19 23
30 23 21 19 7	19 10 8 14 10	6 6 8 10 19	9 13 13 12 20	12 20 17 20 20	18 23 17 20 25
12 11 14 11 17	13 12 13 17 17	18 16 15 16 12	12 14 15 15 16	11 19 13 17 19	18 25 22 15 13
18 21 21 29 21	17 22 18 27 13	10 8 11 24 21	10 19 12 16 19	21 8 15 19 10	20 19 15 16 20
N 100	100	100	100	100	100
A 16.37	17.14	15.04	15.86	18.88	17.58
B ±6.166	±6.863	±6.898	±5.829	±7.115	±5.643
C 75.33%	80.08%	91.72%	73.51%	75.37%	64.20%

SLIDE 25	SLIDE 26	SLIDE 27	SLIDE 28	SLIDE 29	SLIDE 30
2 5 4 3 9	6 6 6 11 3	13 13 8 15 8	4 9 6 5 14	5 5 4 9 8	3 6 5 8 8
12 9 7 12 14	11 8 6 4 7	14 10 8 14 15	10 12 12 8 13	6 7 7 8 3	10 7 7 11 6
8 10 12 9 7	9 12 13 7 5	10 18 12 15 9	16 15 19 15 20	9 12 9 10 12	6 5 9 11 10
17 21 12 17 13	10 9 13 13 8	10 7 16 11 14	10 24 19 19 9	10 18 13 11 15	11 4 18 9 13
18 17 21 19 31	15 13 17 6 13	10 6 2 5 13	18 19 21 22 40	13 24 21 22 24	16 11 21 11 24
32 33 23 24 24	16 25 12 22 14	29 22 21 22 29	17 21 27 30 26	29 15 22 16 14	11 25 22 20 20
25 15 25 25 28	16 7 19 13 16	32 27 37 38 37	28 25 35 39 29	12 26 13 18 17	9 9 12 8 12
16 23 23 25 36	12 9 14 19 19	40 30 33 26 24	44 24 32 36 27	27 13 31 29 21	12 8 18 28 26
31 29 32 31 28	26 32 21 24 32	26 37 22 18 11	23 26 28 30 28	22 26 16 21 12	18 14 17 10 21
36 44 33 48 36	22 21 33 25 17	16 15 21 8 9	33 27 21 23 11	21 11 12 19 16	18 30 31 32 43
37 44 40 38 39	15 42 61 33 32	18 17 12 12 23	8 8 17 9 12	29 23 39 33 22	28 26 34 24 41
13 32 47 28 37	33 27 38 31 44	16 23 22 15 21	21 19 14 10 13	28 23 30 25 17	44 32 37 31 28
29 35 29 19 25	45 41 49 24 25	22 26 21 26 22	18 18 25 27 22	26 19 23 22 19	19 22 19 19 21
28 14 26 26 11	25 18 33 21 25	29 22 20 23 37	22 29 17 19 14	21 21 14 18 20	18 16 13 14 10
10 8 13 11 12	22 24 21 31 25	36 33 26 17 18	12 21 14 34 23	16 21 14 14 15	20 19 18 19 16
17 13 16 11 14	20 24 20 16 22	9 5 2 11 2	21 17 20 11 18	19 24 10 6 16	19 13 11 11 10
18 6 6 14 8	17 17 13 15 18	5 5 13 13 11	13 10 10 13 11	12 18 18 21 15	19 8 12 9 6
16 14 7 7 5	18 13 9 16 16	8 11 10 10 15	15 10 7 12 12	19 16 23 24 19	26 20 28 17 17
10 6 9 4 4	17 11 11 11 8	17 13 11 20 12	25 16 19 14 23	20 16 15 11 13	18 18 20 18 16
9 10 4 17 16	5 16 16 11 13	24 20 11 14 22	19 17 18 16 10	7 5 8 5 9	16 18 10 13 5
N 100	100	100	100	100	100
A 19.46	18.85	17.57	18.92	16.75	16.90
B ±11.425	±10.798	±9.009	±8.206	±7.120	±8.778
C 117.42%	114.57%	102.55%	86.74%	85.01%	103.88%

SLIDE 31	SLIDE 32	SLIDE 33	SLIDE 34	SLIDE 35a	SLIDE 36a
17 6 11 13 8	17 19 13 13 17	7 4 14 6 10	8 5 13 16 17	6 5 9 8 9	9 7 16 10 13
14 7 14 9 17	9 18 16 6 12	9 8 13 9 21	15 10 12 11 16	6 10 9 15 11	19 16 17 16 13
13 13 10 15 18	12 13 16 20 15	9 9 12 9 7	15 9 18 18 14	7 10 17 13 16	23 9 11 25 15
12 19 11 17 22	5 9 10 9 15	13 10 9 7 9	18 22 13 8 13	19 14 16 8 21	10 20 18 19 24
26 19 27 19 19	9 12 6 13 8	14 14 11 6 12	14 3 7 1 5	16 11 12 13 15	22 29 26 27 27
18 23 28 32 30	13 12 10 7 7	10 11 15 14 7	6 8 9 10 18	17 16 10 11 8	28 32 25 25 29
24 26 34 39 53	10 5 10 7 21	7 18 12 14 15	8 16 16 13 19	21 12 21 15 20	15 26 25 30 18
24 33 17 19 15	19 20 22 22 15	21 15 21 13 18	22 17 21 19 23	23 18 22 20 20	24 28 34 23 23
17 21 22 24 26	18 19 18 21 14	30 20 23 18 26	17 16 14 21 20	18 20 24 12 18	25 16 7 21 20
10 17 16 14 17	14 14 19 13 12	12 24 16 12 15	18 17 21 15 16	15 12 17 10 13	12 26 17 17 16
17 28 21 16 29	8 10 15 14 37	12 27 19 13 11	16 15 24 13 18		
35 20 24 61 30	39 25 14 22 15	8 16 18 17 13	16 7 15 19 15	SLIDE 35b	SLIDE 36b
9 14 32 31 24	18 13 16 12 13	16 25 16 22 19	13 18 12 13 12	6 12 6 15 16	10 10 13 11 12
31 20 16 19 17	10 17 19 16 15	12 21 29 22 13	28 26 26 30 34	4 13 19 9 10	6 11 9 6 14
17 14 6 20 17	10 12 18 29 17	16 12 12 16 15	26 22 21 35 29	8 8 8 8 7	13 18 10 4 5
26 19 25 5 8	13 13 5 20 12	6 19 11 15 13	19 31 24 33 37	12 7 7 10 19	11 7 9 6 8
7 2 7 13 23	8 7 10 10 16	12 8 10 11 12	24 33 25 23 18	8 8 9 18 11	5 12 3 6 6
11 13 13 13 12	9 8 11 14 30	6 6 10 2 10	29 19 34 16 28	6 13 10 12 13	11 10 17 12 22
4 12 17 8 7	18 18 12 23 19	7 7 3 7 2	25 30 32 35 29	14 12 21 22 20	26 36 13 14 25
7 6 6 11 11	13 25 17 16 24	8 9 0 9 17	30 35 13 9 11	20 18 30 20 25	30 27 13 15 18
				5 7 8 5 13	21 21 17 10 11
				7 6 8 9 6	5 9 6 15 13
N 100	100	100	100	100	100
A 18.29	14.71	12.99	18.46	12.97	16.45
B ±9.666	±5.825	±5.932	±8.140	±5.525	±7.803
C 105.70%	79.20%	91.34%	88.20%	85.20%	94.88%

N = number of microscopic fields counted.
A = mean number of bacteria per microscopic field.
B = standard deviation of the counts of individual fields.
C = coefficient of variation of the counts of individual fields.

APPENDIX II

Table of data obtained by plating

EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	EXPERIMENT 4	EXPERIMENT 5
Dilution 10^5	Dilution 10^6	Dilution 10^5	Dilution 10^6	Dilution 10^5
60*44 54 70 49 50 62 56 48 57 52 52 37 47 44 40 53 59 57 48 49 45 40 37 48 44 40 56 52 56 67 51 47 46 57 39 62 64 59 54 53 51 46 49 58 51 46 53 63 35 38 46 48 51 50 44 51 57 56 50 58 49 54 43 28 41 42 60 48 61 43 41 36 49 48 46 50 51 31 43 44 47 54 36 32 43 48 43 34 43 43 43	120 107 135 134 115 131 137 133 130 166 103 124 104 148 131 98 132 119 116 121 113 126 137 157 182 150 148 182 144 155 133 178 146 155 130 239 130 132 117 115 177 88 162 154 176 141 176 140 119 146 154 146 128 121 159 147 153 173 136 160 142 183 141 153 123 133 135 126 121 220 146 140 127 151 167 120 131 121 197 184 104 128 136 165 113 126 127 132 147 127 163 138 141 149 140 124 155 152 121 139 135 147 129 175 149 114 154 146 186	151 160 152 132 186 167 155 126 158 163 139 192 193 212 125 170 150 196 151 182 188 199 134 167 174 176 184 174 173 179 178 179 161 220 227 215 180 164 169 165 163 174 195 178 121 182 163 148 207 181 152 172 153 178 166 193 132 173 155 123 137 97 167 81 173 124 134 105 131 123 118 134 144 121 162 176 219 155 178 191 162 206 193 209 169 165 206 188 183 202 203 210 163 193 164 191 107 216 201 173	65 54 52 36 37 64 55 49 39 79 43 47 40 53 52 56 46 62 52 45 59 64 60 49 62 58 58 40 53 53 57 69 51 74 68 53 53 61 51 58 56 57 57 56 51 79 50 54 57 78 58 51 73 62 49 60 50 52 58 55 59 51 51 56 49 59 45 53 58 58 49 62 61 46 54 41 63 64 48 41 44 60 44 56 61 54 57 62 44 56 46 53 54 51 46 38 74 62 56 47	295 215 284 251 212 226 208 273 185 178 221 225 236 238 241 225 171 223 255 213 230 198 208 263 257 177 144 221 224 256 200 224 258 244 240 210 162 203 229 282 184 213 202 215 218 251 226 235 147 186 130 182 199 266 278 212 255 238 188 174 155 178 289 250 212 242 204 232 210 250 172 212 255 226 281 223 191 182 180 207 255 165 176 282 200 235 274 174 198 183 252 182 167 180 227 151 182 131 194 199 203 190 208 210
N 92 A 48.70 B ±8.178 C 33.58% D ±0.857 E 3.52% F 47.0–50.4	109 141.76 ±24.194 34.14% ±2.328 3.28% 137.10–146.42	100 167.27 ±29.274 35.00% ±2.942 3.52% 161.9–173.15	100 54.75 ±8.726 31.88% ±0.877 3.20% 53.00–56.50	104 215.13 ±36.45 33.89% ±3.592 3.34% 207.95–222.31

EXPERIMENT 3a	EXPERIMENT 3b	EXPERIMENT 4a	EXPERIMENT 4b	EXPERIMENT 5a	EXPERIMENT 5b
Dilution 10^7	Dilution 10^8	Dilution 10^7	Dilution 10^6	Dilution 10^7	Dilution 10^6
128*195 165 127 94 133 129 121 112 139	295 222 194 168 211 163 232 162 159 217	69 69 68 79 81 90 81 80 72 78	287 307 340 332 421 309 327 310 320 358 302 304	40 43 45 39 41 45 48 46 43 55 39 36 46	122 95 146 128 117 142 140 131 141 167 141 123 119 162 166
N 10 A 134.3 D ±2.726 E 4.06% F 128.8–139.8	10 202.3 ±13.317 13.36% 175.3–229.3	10 76.70 ±2.726 7.11% 71.3–82.1	12 326.41 ±10.24 6.27% 305.8–346.9	13 43.54 ±2.361 10.84% 38.82–48.26	15 136.00 ±5.129 7.54% 125.74–146.26

EXPERIMENT 6	EXPERIMENT 7	EXPERIMENT 8	EXPERIMENT 9	EXPERIMENT 10	EXPERIMENT 11	EXPERIMENT 12
Dilution 10^4	Dilution 10^4	Dilution 10^5	Dilution 10^5	Dilution 10^5	Dilution 10^5	Dilution 10^4
114*102 110 78	265 234 214 210	45 34 30 48	79 70 80 79	79 86 90 80	99 88 98 81	110 114 101 112
A 101.00 D ±4.72 E 9.35% F 91.6–110.4	230.75 ±4.72 4.09% 221.3–240.2	39.25 ±4.72 24.05% 29.8–48.7	77.00 ±4.72 12.26% 67.6–86.4	83.75 ±4.72 11.27% 74.3–93.2	91.50 ±4.72 10.32% 82.1–100.9	109.25 ±4.72 8.64% 99.8–118.7

APPENDIX II—*Concluded*

Table of data obtained by plating—Concluded

EXPERIMENT 13	EXPERIMENT 14	EXPERIMENT 15	EXPERIMENT 16	EXPERIMENT 17a	EXPERIMENT 17b
Dilution 10^5	Dilution 10^5	Dilution 10^5	Dilution 10^5	Dilution 10^5	Dilution 10^5
124*117 125 119	148 156 141 142	172 169 175 183	167 218 202 170	332 469 367 441 437 368 448 383 438 336 372	403 360 391 363 348 301 342 314 368 318
N 4	4	4	4	11	10
A 121.25	146.75	174.75	189.25	399.18	350.8
D ±4.72	±4.72	±4.72	±4.72	±14.60	±10.47
E 7.78%	6.43%	5.40%	4.99%	7.31%	5.97%
F 111.8–130.7	137.3–156.2	165.3–184.2	179.8–198.7	370.0–428.4	329.9–371.7

N = number of plates.
A = mean number of colonies per plate.
B = standard deviation of individual plate counts.
C = coefficient of variation of individual plate counts.
D = standard deviation of the means.
E = coefficient of variation of the means.
F = the range which will include 97 per cent of the means.
* Number of colonies per plate.

APPENDIX III

Table of dilution data

	EXPERIMENT 1			EXPERIMENT 2			EXPERIMENT 3			EXPERIMENT 4			EXPERIMENT 5		
Dilution....	10^8	10^7	10^6	10^9	10^8	10^{10}	10^7	10^8	10^9	10^7	10^8	10^9	10^6	10^7	10^8
Set	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3
1	10	4	1	10	1	0	10	4	1	10	3	0	10	8	3
2	10	3	0	7	1	0	10	1	0	10	6	0	10	9	1
3	10	5	1	10	3	0	9	3	1	10	4	1	10	9	2
4	10	3	0	8	2	0	10	2	0	10	2	0	10	10	2
5	10	5	0	9	2	0	10	3	1	10	4	0	10	9	2
6	9	3	1	9	1	0	10	2	1	10	5	0	10	10	2
7	9	5	0	10	2	0	10	4	0	10	3	1	10	9	4
8	10	4	0	9	0	0	9	2	1	10	5	0	10	7	1
9	10	5	0	5	3	0	10	3	0	10	3	1	10	9	4
10	10	2	0	7	1	0	10	2	0	10	5	0	10	8	4
11	10	3	1	7	1	0	10	3	0	10	5	1	10	9	3
12	10	4	0	5	1	0	10	1	0	10	4	2	10	8	2
13	10	3	1	7	4	0	9	2	0	10	4	0	10	8	2
14	10	6	1	9	2	0	10	4	1	10	6	0	10	9	5
15	10	4	2	9	4	0	10	4	0	10	4	0	10	10	4
16	10	2	0	8	4	0	10	4	1	10	3	1	10	8	3
Total.....	158	61	8	129	32	0	157	44	7	160	66	7	160	140	44
A	0.4669			0.1716			0.3642			0.5488			2.3407		
B	0.389–0.584			0.1350–0.2146			0.3035–0.4550			0.4570–0.6860			1.950–2.950		

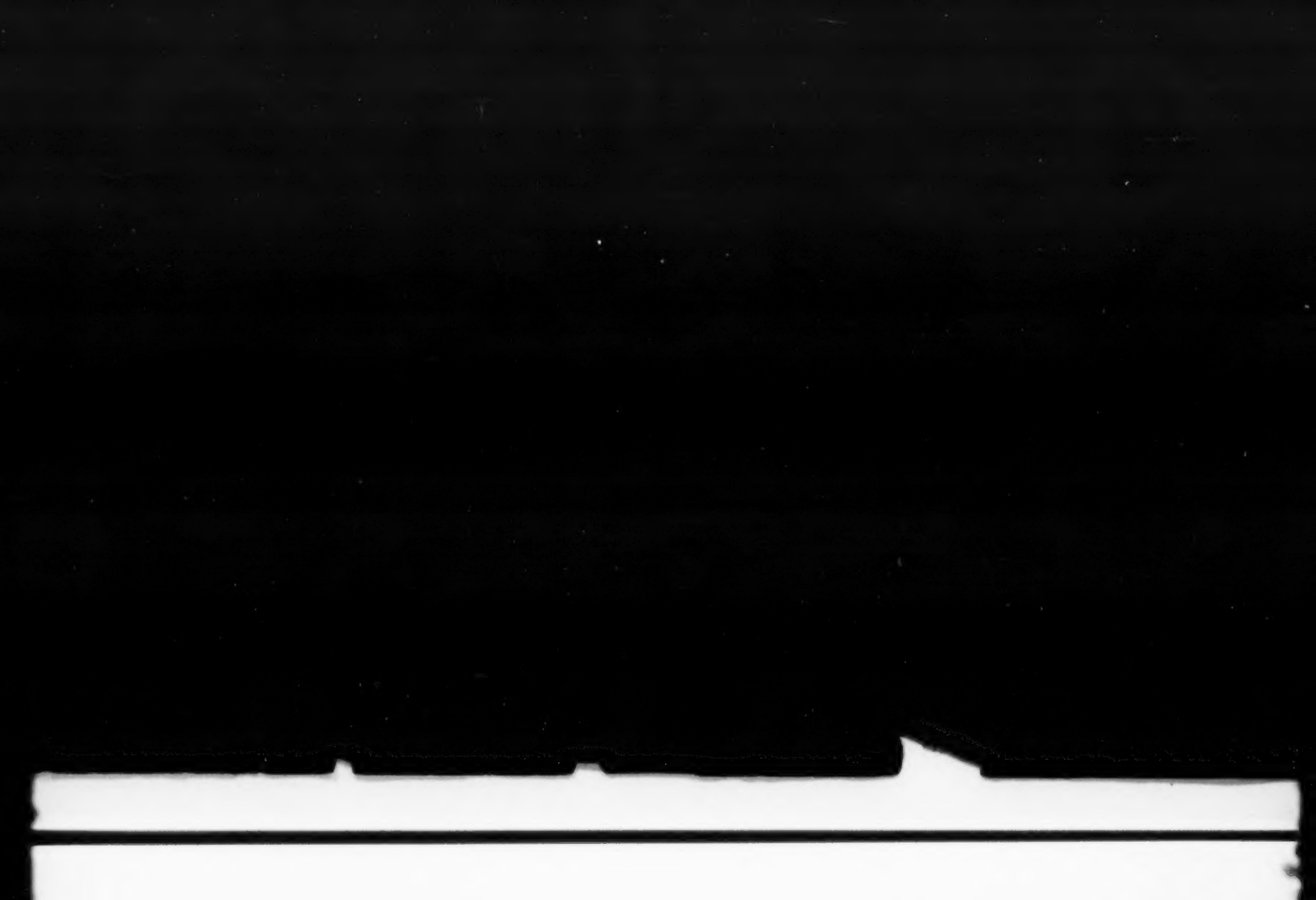
	EXPERIMENT 3a			EXPERIMENT 3b			EXPERIMENT 4a			EXPERIMENT 4b			EXPERIMENT 5a			EXPERIMENT 5b		
Dilution....	10^9	10^{10}	10^{11}	10^8	10^9	10^{10}	10^9	10^8	10^{10}	10^8	10^9	10^{10}	10^9	10^9	10^{10}	10^7	10^8	10^9
Set	p_1	p_2	p_3	p_1	p_2	p_4	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3
1	7	1	0	10	3	0	10	6	1	9	1	0	9	5	1	10	9	1
2	8	0	0	10	1	1	10	7	1	10	3	0	9	5	1	10	10	2
3	7	0	1	10	3	0	10	7	0	8	1	0	10	2	0	10	10	1
4	6	1	0	10	7	0	10	7	0	10	3	0	10	2	1	10	8	3
Total.....	28	2	1	40	14	1	40	27	2	37	8	0	38	14	3	40	37	7
A	0.1118			0.4650			1.0308			0.2404			0.3786			2.3924		
B	0.0760–0.1803			0.3141–1.453			0.7012–1.6626			0.1635–0.3877			0.2576–0.6106			1.6275–3.8387		

	EXPERIMENT 6			EXPERIMENT 7			EXPERIMENT 8			EXPERIMENT 9			EXPERIMENT 10			EXPERIMENT 11		
Dilution....	10^5	10^6	10^7	10^6	10^7	10^8	10^8	10^7	10^9	10^6	10^7	10^8	10^6	10^7	10^8	10^7	10^8	10^9
Set	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3
1	10	7	3	9	2	1	10	4	0	10	7	0	10	5	1	4	0	0
2	10	6	0	10	2	0	10	3	0	10	5	2	10	6	2	6	1	0
3	10	5	0	8	1	1	10	2	0	10	5	1	10	4	0	9	0	0
4	10	5	0	9	5	0	10	3	0	10	6	1	10	6	1	5	0	0
Total.....	40	23	3	36	10	2	40	12	0	40	23	4	40	21	4	24	1	0
A	0.8469			0.2526			0.3991			0.8833			0.7850			0.0781		
B	0.5761–1.3660			0.1720–0.4077			0.2715–0.6437			0.6009–1.4247			0.5340–1.2661			0.0531–0.1260		

APPENDIX III—*Concluded*
Table of dilution data—Concluded

	EXPERIMENT 12			EXPERIMENT 13			EXPERIMENT 14			EXPERIMENT 15			EXPERIMENT 16			EXPERIMENT 17		
Dilution....	10^7	10^8	10^9	10^7	10^8	10^9	10^7	10^8	10^9	10^7	10^8	10^9	10^7	10^8	10^9	10^5	10^6	10^7
Set	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3	p_1	p_2	p_3
1	6	1	0	9	1	0	8	0	0	6	1	1	8	1	0	10	4	1
2	7	1	0	9	0	1	6	1	0	8	2	0	10	1	0	10	5	0
3	7	1	0	8	0	0	8	1	0	8	0	0	9	0	0	10	6	0
4	7	0	0	8	2	0	9	2	0	9	0	0	9	1	0	10	5	1
Total.....	27	3	0	34	3	1	31	4	0	31	3	1	36	3	0	40	20	2
A	0.0949			0.1674			0.1388			0.1385			0.1861			0.6800		
B	0.0646–0.1531			0.1139–0.2700			0.0944–0.2239			0.0942–0.2234			0.1266–0.3002			0.4636–1.0968		

p = the number of tubes which showed growth.
A = the most probable number of bacteria per cubic centimeter of the middle dilution.
B = the range which will include 97 per cent of the data.



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